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(73)

**SYNGENTA PARTICIPATIONS AG**  
**Schwarzwaldallee 215 CH-4058 BASEL XX (CH).**

(72)

**HARMS, CHRISTIAN T. (US).**  
**HORN, MICHAEL E. (US).**  
**SHILLITO, RAYMOND D. (US).**

(74)

**FETHERSTONHAUGH & CO.**

(54) REGENERATION DE PLANTES GRAMINEES DE LA SOUS-FAMILLE DES POOIDEAE A PARTIR DE PROTOPLASTES

(54) REGENERATION OF GRAMINACEOUS PLANTS OF THE SUBFAMILY POOIDEAE FROM PROTOPLASTS

(57)

The present invention provides cell cultures derived from graminaceous plants which cultures are capable of being regenerated into plants, including fertile plants. Methods of accomplishing this regeneration are also provided. In addition, a novel procedure for the cryopreservation of embryogenic cell cultures is described.



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(19) (CA) **CANADIAN PATENT** (12)

(54) Regeneration of Gramineous Plants of the Subfamily Pooideae From Protoplasts

(72) HORN, MICHAEL E., U.S.A.  
HARMS, CHRISTIAN T., U.S.A.  
SHILLITO, RAYMOND D., U.S.A.

(73) SYNGENTA PARTICIPATIONS AG, SWITZERLAND

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REGENERATION OF GRAMINACEOUS PLANTS OF THE SUBFAMILY POOIDEAE FROM  
PROTOPLASTS

ABSTRACT OF THE INVENTION

The present invention provides cell cultures derived from graminaceous plants which cultures are capable of being regenerated into plants, including fertile plants. Methods of accomplishing this regeneration are also provided. In addition, a novel procedure for the cryopreservation of embryogenic cell cultures is described.

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REGENERATION OF GRAMINACEOUS PLANTS OF THE SUBFAMILY POOIDEAE FROM  
PROTOPLASTS

The present invention relates to graminaceous plants of the subfamily of the *Pooideae* that are regenerated from protoplasts or from protoplasts with regenerated cell walls (plant cells) or from protoplast-derived calli and to generally applicable methods for regeneration of these plants. It also relates to the embryogenic cell cultures (suspension cultures or callus cultures) and calli that constitute the source for protoplasts which can be regenerated into plants. It further relates to the methods of producing the embryogenic cell cultures mentioned above, to the cryopreservation of said embryogenic cell cultures and of the embryogenic calli, and to transgenic *Pooideae* plants regenerated from genetically modified protoplasts.

Most of the plant species upon which Mankind is dependent for the bulk of its diet belong to that group of plants known collectively as the *Gramineae*. The *Gramineae* (*Poaceae*) are, from a commercial point of view, the most important family within the class of monocotyledonous plants. The *Gramineae* embrace, for example, the following subfamilies and genera:

<u>Subfamily</u>	<u>Genus within the subfamily</u>
<i>Bambusoideae</i>	<i>Bamboo</i>
<i>Andropogonoideae</i>	<i>Saccharum</i> [sugarcane]
	<i>Sorghum</i>
	<i>Zea</i> [corn]
<i>Arundineae</i>	<i>Phragmites</i>
<i>Oryzoideae</i>	<i>Oryza</i> [rice]
<i>Panicoideae</i>	<i>Panicum</i> (*)
	<i>Pennisetum</i> (*)

<i>Pooideae (Festuciadeae)</i>	<i>Setaria</i>	(*)
	<i>Poa</i>	(**)
	<i>Festuca</i>	(**)
	<i>Lolium</i>	(**)
	<i>Bromus</i>	(**)
	<i>Trisetum</i>	(**)
	<i>Agrostis</i>	(**)
	<i>Phleum</i>	(**)
	<i>Dactylis</i>	(**)
	<i>Alopecurus</i>	(**)
	<i>Avena</i>	[oats] (^)
	<i>Triticum</i>	[wheat] (^)
	<i>Secale</i>	[rye] (^)
	<i>Hordeum</i>	[barley] (^)

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\* (millets)

\*\* (grasses)

^ (small grain cereals)

Among the subfamilies of the *Gramineae* the subfamily *Pooideae* is a group of economically highly important plants that includes, for example, the two closely related subgroups consisting of the grasses and the small grain cereals.

Interestingly these *Pooideae* plants have also been the most difficult to manipulate scientifically. Until now, no generally applicable method is known for the regeneration of *Pooideae* plants, or fertile *Pooideae* plants, or for *Pooideae* plants containing stably incorporated exogenous DNA from protoplasts, although plant regeneration from cultured protoplasts is essential for the application of somatic hybridization and the production of transgenic plants via direct gene transfer. The present state of the art in gene transfer into cereals has recently been reviewed by Cocking and Davey, (1987).

Sources for cereal cultures, protoplasts, the isolation of cereal protoplasts, and their properties are reported, for example, in the following book: ["Cereal Tissue and Cell Culture" Bright, S.W.J. and Jones, M.G.K., (eds) (1985) Nijhoff, M., Junk, W., Dordrecht].

Stable transformation has been already achieved in the *Gramineae* by chemically and electrically stimulated uptake of DNA into protoplasts ("direct gene transfer") [Potrykus *et al*, 1985; Loerz *et al*, 1985; Fromm *et al*, 1986], but plant regeneration was not possible from the lines used in these studies.

So far, graminaceous plants have only been successfully regenerated from protoplasts other than of the subfamily *Pooideae*: For example, Abdullah *et al*, (1986), report the efficient plant regeneration from rice (sub-family: *Oryzoideae*) protoplasts through somatic embryogenesis. Yamada *et al*, (1986) also describe rice plant regeneration from protoplast-derived calli. Also Rhodes *et al*, (1988) describe the regeneration of non-fertile plants of maize. Cocking and Davey (1987) discuss the present state of the art in gene transfer in cereals.

The regeneration of graminaceous plants of the subfamily *Pooideae* from tissue cultures is known. Hanning *et al*, (1982) describe embryo and plantlet formation from leaf segment-derived callus of *Dactylis glomerata* L.

Some further examples of regeneration of *Pooideae* plants from cultured cells are reported in the following articles:

*Lolium rigidum*: Skene *et al*, 1983.

*Lolium perenne*, *Lolium multiflorum*: Ahloowalia, 1975.

*Lolium multiflorum*, *Festuca arundinacea*: Kasperbauer *et al*, 1979.

*Alopecurus arundinaceus*, *Agropyron crystatum*, *Stipa viridula*, *Bromus inermis*, *Agropyron smithii*: Lo *et al*, 1980.

*Agrostis palustris*: Krans *et al*, 1982.

The state of tissue culture in forage grasses has also been reviewed by Ahloowalia (1984).

However, these *Pooideae* plants were not regenerated in these cases from the type of starting material described in the present application but from other types of cell cultures. It has not been demonstrated in the above examples that regeneration was *de-novo* by way of somatic embryogenesis. The above quoted reference did not comprise the isolation and culture of protoplasts or the regeneration of plants from protoplasts.

Although there has been great interest in genetic transformation and regeneration of graminaceous plants in the subfamily *Pooideae*, there has been no description to date of a successful in-vitro method which can lead to regenerated, optionally transformed, protoplast-derived, plants or fertile plants (Cocking and Davey, 1987).

Until now all investigations and every effort made in this direction failed, in so far as they resulted in embryos or at most in non-viable plantlets that died in an early stage and therefore could not be successfully transferred to soil (Ahloowalia, 1984).

No description of a procedure for producing *Pooideae* protoplasts capable of undergoing differentiation to plants and preferably to whole fertile plants, much less of the regeneration of *Pooideae* plants from protoplasts or protoplast-derived calli, has appeared.

These on other objectives have been achieved in accordance with the present invention which provides a method for producing protoplasts that can form cell and callus colonies. The protoplasts can, if desired, be transformed, and the resultant calli are capable of being regenerated into *Pooideae* plants. The process for producing protoplasts capable of dividing and forming callus, which then can be regenerated into plants, requires as a starting material novel embryogenic cell cultures (suspension cultures or callus cultures) or embryos. Such embryogenic cell cultures, embryos and methods for producing and identifying them will be described, and are considered part of the invention. Embryogenic callus from which the suspensions are derived can also be used as a starting

material for protoplasts. Such callus and suspensions, embryos and methods for producing and identifying them will be described, and are also considered part of the present invention.

These embryogenic cultures are the source of protoplasts capable of being transformed with exogenous DNA, and of dividing and forming callus, which then can be regenerated into whole plants, including whole, fertile plants that can grow in soil.

One could not predict from the prior art at the time this invention was made, that graminaceous plants, particularly fertile graminaceous plants, of the subfamily *Pooideae* could be regenerated from protoplasts, from protoplast-derived cells or protoplast-derived calli.

Even less predictable was that *Pooideae* protoplasts containing stably incorporated into their genome exogenous DNA could also be regenerated into transgenic plants, particularly into fertile transgenic plants.

This invention is, therefore, primarily directed to embryogenic cell cultures (suspension cultures or callus cultures) derived from graminaceous plants of the subfamily *Pooideae* from which protoplasts can be isolated, wherein the protoplasts regenerate cell walls, divide and form callus capable of being regenerated into plants, including fertile plants.

This invention also relates to *Pooideae* protoplasts and the resulting plant cells (after regeneration of the cell walls) that can be regenerated into plants which are preferably fertile, preferably to such protoplasts derived from cell cultures or from embryogenic cell suspensions.

This invention also relates to plant cells, calli, embryogenic cell suspensions, embryos, plantlets and plants derived from said protoplasts.

Furthermore, this invention relates to the regenerated *Pooideae* plants and propagules thereof, especially to those derived from protoplasts or plant cells containing stably incorporated into their genome exogenous



DNA expressible in plants. Propagules include any plant material that can be sexually or asexually propagated or propagated in-vivo or in-vitro. Among this material protoplasts, cells, calli, tissues, organs, zygotes,  
5 embryos, pollen or seeds obtained from transgenic *Pooideae* plants are preferred. The progeny of said *Pooideae* plants, including mutants and variants thereof, including those of plants obtained from somatic cell fusion, genetic modification, or mutant selection, are further objectives of  
10 this invention.

This invention also relates to a method of producing *Pooideae* protoplasts and *Pooideae* plant cells that can be regenerated into plants, particularly into fertile plants, moreover, to a method of producing *Pooideae* calli  
15 derived from said protoplasts or plant cells and being capable of regeneration into plants, preferably into fertile plants. In addition, it relates to a method of regenerating *Pooideae* plants, from these calli. These methods are described in detail hereinafter.

20 This invention thus relates to a method of producing protoplasts of graminaceous plants of the subfamily *Pooideae*, which protoplasts are capable of being regenerated into plants, which process comprises: (a) isolating tissue from suitable parts of *Pooideae* plants, (b)  
25 culturing this tissue in a medium capable of inducing the formation of embryogenic callus and of embryos, (c) periodically subculturing the embryogenic callus and embryos on fresh medium capable of sustaining continuous proliferation, (d) isolating embryogenic cell clusters  
30 after 0 to 500 transfers, and (e) removing the cell walls with suitable enzyme mixtures and isolating the resultant protoplasts.

This invention also relates to a method of producing a cell culture of a graminaceous plant of the subfamily *Pooideae*, which cell culture is capable of being regenerated into plants, which method comprises: (a)

- 5 culturing in a suitable culture medium protoplasts capable of being regenerated into plants until they form cell colonies, and (b) culturing said cell colonies or parts thereof on a medium suitable to promote cell culture formation, and (c) isolating the resultant cell culture.

- 10 This invention also relates to a method of regenerating graminaceous plants of the subfamily *Pooideae* from callus comprising: (a) culturing callus of *Pooideae* plants, which callus is derived from protoplasts and is capable of being regenerated into plants on a medium capable  
15 of inducing embryo formation until embryos are formed, (b) culturing the embryos on a medium suitable to induce them to mature and germinate, and (c) culturing the resultant plantlets until sufficiently developed to be transferred to soil to form mature plants.

- 20 This invention also relates to a method of regenerating fertile graminaceous plants of the subfamily *Pooideae* from callus, which process comprises: (a) culturing callus of *Pooideae* plants, which callus is derived from protoplasts and is capable of being regenerated into fertile  
25 plants on a medium capable of inducing embryo formation until embryos are formed, (b) culturing the embryos on a medium suitable to induce them to mature and germinate, (c) culturing the resultant plantlets until sufficiently developed to be transferred to soil to form mature plants,  
30 and (d) obtaining seed following controlled or open pollination.

These and further objects of the present invention will become available from the following detailed description.

DESCRIPTION OF THE FIGURES

5           Figure 1: Shows protoplast-derived colonies of *Dactylis glomerata* L. growing in an agarose bead suspended in liquid medium.

          Figure 2: Shows a plantlet arising from protoplast-derived callus of *Dactylis glomerata* L. growing  
10 on SH-O medium.

          Figure 3: Shows rooted plantlet from protoplast-derived callus of *Dactylis glomerata* L. growing on SH-O medium in a container.

          Figure 4: Shows a plant of *Dactylis glomerata* L.  
15 regenerated from protoplasts (on left) together with a wild type *Dactylis glomerata* L. plant (on right).

Figure 5: Shows the plasmid pCIB709 that can be used for transformation of *Dactylis glomerata* L. protoplasts to confer resistance to hygromycin. Plasmid pCIB709 has been deposited in accordance with requirements of the Budapest Treaty with the American Type Culture Collection (ATCC) Rockville, Maryland, USA and has accession number ATCC 40428. The date of deposition is February 12, 1988.

Legend: 35S prom: 35S promoter region  
Hygro-gene: Hygromycin phosphotransferase (APH type IV) structural gene.  
35S term: region of CaMV containing the  
3' polyadenylation site of the 35S transcript of CaMV.

Figure 6: Shows the Southern analysis of DNA from different *Dactylis glomerata* L. calli recovered after transformation of protoplasts with pCIB709. The DNA was probed with the XbaI-SstI fragment of pCIB709.

Lanes 1,2: 10 and 2ng pCIB709 cut with restriction endonuclease BamHI.

Lanes 4-8: DNA from *Dactylis glomerata* L. callus cultures recovered after transformation of protoplasts with pCIB709, cut with BamHI.

Lanes 9,17: DNA from control untransformed *Dactylis glomerata* L. callus derived from protoplasts with BamHI.

Lanes 10-13: DNA from *Dactylis glomerata* L. callus cultures recovered after transformation of protoplasts with pCIB709, cut with BamHI.

Lane 14: DNA from *Dactylis glomerata* L. callus cultures recovered after transformation of protoplasts with pCIB709, cut with BamHI.

Lanes 15,16: DNA from *Dactylis glomerata* L. callus cultures recovered after transformation of protoplasts with pCIB709, cut with BamHI.

Lane 4: is empty.

The DNA in lanes 6, 10, 12, and 15 shows the presence of foreign DNA integrated into the genome of the *Dactylis glomerata* L. cells as evidenced by the darkening of the film. The 1063bp fragment expected from BamHI digestion of the integrated hygromycin gene of pCIB709 (nucleotides 583-1646 of pCIB709) is indicated by the arrow.

#### DEFINITIONS

In order to provide a clear and consistent understanding of the specification and the claims, including the scope given to such terms, the following definitions are provided:

Plant Cell: The structural and physiological unit of plants, consisting of a protoplast and the cell wall.

Plant Tissue: A group of plant cells organized into a structural and functional unit.

Plant Organ: A distinct and visibly differentiated part of a plant such as root, stem, leaf, flower bud, or embryo. A plant organ may consist of different types of plant cells or plant tissues.

Protoplast: Isolated plant cell without a cell wall.

Cell Culture: Proliferating mass of cells in an undifferentiated or partially differentiated state.

Embryo: A minute early developmental stage of a plant, either derived from a zygote (sexual embryo) or from an embryogenic somatic cell (somatic embryo), with stages of recognizable morphology, structure and cellular organization comprising cellular to globular to cotyledonary

1341473

stages [Corn embryo development is, for example, described in Randolph (1936); grass embryo development is, for example, described in Brown (1960)].

Cell cluster: A group of interconnected cells attached to each other; usually derived from one or a few ancestral cells or protoplasts by cell division.

Plantlet: A multicellular structure made up of a shoot and root in the form of a small plant.

Dicamba: 3,6-dichloro-2-methoxy benzoic acid.

MES: 2-[N-morpholino]ethane sulfonic acid.

2,4-D: 2,4-dichlorophenoxyacetic acid.

Picloram: 4-amino-3,6,6-trichloropicolinic acid.

Tris-HCl: alpha,alpha,alpha-tris(hydroxymethyl)methylamine hydrochloride.

EDTA: 1-ethylendiamine N,N,N',N'-tetraacetic acid.

PEG: polyethylene glycol

Agarose: Preparation and purification of agarose are described, for example by Guiseley and Renn, (1975). Agarose is one of the constituents of agar. Commercially available agar normally consists of a mixture of neutral agarose and ionic agaropectin with a large number of side groups. Commercial agarose is normally obtained from agar by conventional methods. Usually a certain number of side chains remains intact and determines the physicochemical properties of the agarose, such as gel formation and melting temperature. Low-melting agarose, especially SeaPlaque® agarose, is a preferred solidifying agent within the process described hereinafter.

SH-0 medium: Medium of Schenk and Hildebrandt (1972); without hormones. (SH medium can be liquid or solidified with 0.8 % (w/v) agar or with 0.5 % (w/v) GelRite®). The medium is normally sterilized by heat or sterilized by autoclaving at 121°C and 151 lb/in<sup>2</sup> pressure for about 15 to 20 minutes as known in the art.

GelRite®: GelRite Gellan Gum, Scott Laboratories Inc., Fiskersville, RI 02823.

SH-30 medium: SH-0 medium containing 30 µM Dicamba.

SH-45 medium: SH-0 medium containing 45 µM Dicamba.

KM-8p medium: Medium 8p of Kao, (1975).

This medium may be liquid or solidified with agar, agarose or GelRite®, and may equally well be prepared and used without ascorbic acid, vitamin D, and vitamin A. The medium components except solidifying agent are normally sterilized by filtration through a 0.2 µm filter.

RY-2 medium: Medium of Yamada *et al.*, (1986).

OMS medium: Medium of Murashige and Skoog (1962). The medium can be solidified, for example, with 0.8 % (w/v) agar or agarose or with 0.5 % (w/v) GelRite®. For the purposes described in this application, this medium can also be prepared such that it contains the vitamin composition of the B5 medium of Gamborg *et al.*, (1968).

Cellulase RS: Cellulase RS, Yakult Honsha Co. Ltd., 1-1-19 Higashi-Shinbashi, Minato-ku, Tokyo 105, Japan.

Pectolyase Y-23®: Seishin Pharmaceutical Co. Ltd., 4-13 Koami-cho, Nihonbashi, Tokyo, Japan.

Parafilm®: Parafilm® laboratory film - American Can Co. Greenwich, CT, 06830, USA.

Nalgene® filter: Nalge Co., Division of Sybron Corp. Rochester, New York, 14602, USA.

BglIII: Restriction enzyme BglIII; New England Biolabs, 32 Tozer Road, Beverly, MA, 01915, USA, or any other commercial supplier.

BamHI: Restriction enzyme BamHI; New England Biolabs, 32 Tozer Road, Beverly, MA, 01915, USA, or any other commercial supplier.

Casein hydrolysate: Casein Hydrolysate - Enzymatic Hydrolysate from bovine milk, Type 1, Sigma Co. PO. Box 14508, St. Louis, MO 63178, USA.

Hygromycin B: Cytotoxin: Hygromycin B, purified; Cat No. 400050 Calbiochem Behring Diagnostica, La Jolla, CA 92037, USA, Lot No. 702296.

GeneScreen Plus®: Cat. No. NEF 976, NEN Research Products, 549 Albany St., Boston, MA 02118, USA.

TBE buffer: Tris-borate buffer - common buffer for electrophoresis, see Maniatis *et al*, (1982).

Spin column: Sephadex® G25 prepacked column, Cat. No. 100402, Boehringer Mannheim Biochemicals, Piscataway, NJ, USA.

SDS: Sodium dodecyl sulfate.

SSC: 1.54 mM NaCl, 0.154 mM Na citrate as described in Maniatis *et al*, (1982).

CETAB: Hexadecyltrimethylammonium bromide.

IBI Random Primer Kit: 'Prime Time' random primer kit, International Biotechnologies Inc., PO. Box 1565, New Haven, CT 07606, USA (Catalog No. 77800; Lot No. F630-01).



It has now been surprisingly found that graminaceous plants, of the subfamily *Pooideae* can be regenerated from a specific type of protoplasts and from cells or callus derived from these protoplasts.

This regeneration of plants, including fertile plants is also possible if the protoplasts contain exogenous DNA, preferably exogenous DNA which is stably incorporated into the plant genome and capable of being expressed in plants.

Any graminaceous plants, of the subfamily *Pooideae* may be used in the present invention. Preferred, however, are *Pooideae* plants belonging to the grasses, for example, each genus selected from the group consisting of *Poa*, *Festuca*, *Lolium*, *Bromus*, *Trisetum*, *Agrostis*, *Phleum*, *Alopecurus* and *Dactylis*. Most preferred is *Dactylis*. Also preferred are *Pooideae* plants belonging to the small grain cereals, for example, each genus selected from the group consisting of *Avena* (oats), *Triticum* (wheat), *Secale* (rye) and *Hordeum* (barley).

The specific type of *Pooideae* protoplasts that divide and form cell cultures capable of being regenerated into plants originate from cell cultures, preferably embryogenic cell cultures. The embryogenic cell cultures are preferably embryogenic suspensions or embryogenic callus cultures. The cell cultures may be derived from suitable parts of *Pooideae* plants. Suitable parts of plants include but are not limited to the basal parts of young inner leaves, immature sexual embryos, immature inflorescences, mature seeds or seedling tissues of *Pooideae* plants.

Step A: Preparation of embryogenic suspensions from plant tissue

Embryogenic callus is initiated from a suitable part of a *Pooideae* plant, typically from the basal portion of young leaves, most preferably the younger, inner leaves of a *Pooideae* plant. This can be carried out as described for *Dactylis glomerata* L. by Hanning et al, (1982) but can also be used for all other *Pooideae* plants.

The leaves are, for example, sliced in small sections or segments about 1 mm to 5 mm in length or diameter. Form and size of these pieces are not critical. These segments are plated on a suitable callus inducing and maintenance medium and cultured until callus and/or embryogenic structures develop. A suitable medium is, for example SH medium [Schenk and Hildebrandt, 1972] containing 30  $\mu$ M dicamba and 0.8 % (w/v) agar or agarose as a gelling agent. Among other suitable media are those described in George *et al.*, (1987). Callus and/or embryogenic structures usually appear within 2 to 6 weeks after plating. Initiation and maintenance of the callus may be carried out in the light or preferably in the dark and at temperatures between 0°C and 50°C, preferably 20°C and 32°C, most preferably 25°C and 28°C. Embryogenic callus may also be prepared by other methods known in the art, such as those for barley described by Lührs and Lörz (1987) and the references contained therein. These methods can be used for other *Pooideae*.

Embryogenic suspension cultures are initiated by putting fresh pieces of the embryogenic callus into a suitable liquid medium, for example, 0.5 g callus in 50 ml of a liquid medium as described by Gray, *et al.*, (1985) containing 45  $\mu$ M dicamba and 4 g/liter casein hydrolysate. Among other suitable media are those described in George *et al.*, (1987). The suspension cultures are grown at temperatures between 10°C and 40°C, preferably 20°C and 32°C, most preferably between 25°C and 30°C. Succession of light and dark phases during the culturing period can be of advantage. The suspension is preferably grown under a 5 to 20 hours, preferably 16 hours light period followed by a 5 to 20 hours, preferably 8 hours, dark period. The light intensity is typically between 0.1  $\mu$ E/m<sup>2</sup>s and 100  $\mu$ E/m<sup>2</sup>s (E = Einstein; m = meter; s = second), preferably between 30  $\mu$ E/m<sup>2</sup>s and 80  $\mu$ E/m<sup>2</sup>s. Shaking of the suspension during the culturing period is also advantageous. Shaking can be carried out, for example, on a gyratory shaker at about 100 rpm to 150 rpm in Delong flasks sealed with a plastic film that is permeable to light and gases or any other suitable closure. After approximately three to five weeks the larger cellular clumps are allowed to settle for about 30 seconds and the supernatant medium containing only small cell clusters is removed and transferred to fresh medium to initiate new cultures.

This procedure can be repeated periodically, preferably every 3 to 4 weeks, using the most successful cultures as judged by smaller clump size and quality. After 4 to 20 transfers, usually after 6 to 8, transfers, the suspensions are essentially free of non-embryogenic cells and the majority of the embryogenic cell clusters are typically about 150  $\mu\text{m}$  to 2'000  $\mu\text{m}$  in size.

In the procedure for obtaining embryogenic suspensions it is preferred that the suspensions consist predominantly of small pre-embryogenic masses. By subculturing only the upper part of the suspension after allowing the larger material to settle it is possible to enrich significantly the proportion of pre-embryogenic masses.

Thus, one objective of the present invention is the embryogenic cell culture, suspension culture or callus culture, derived from graminaceous plants of the subfamily *Pooideae* from which protoplasts can be isolated, wherein the protoplasts regenerate cell walls, divide and form callus capable of being regenerated into plants and preferably into fertile plants

Embryogenic cell cultures (suspension cultures and callus cultures) derived from grasses, especially from those selected from the genera consisting of *Poa*, *Festuca*, *Lolium*, *Bromus*, *Trisetum*, *Agrostis*, *Phleum*, *Alopecurus* and *Dactylis* are preferred embodiments of the present invention. Mostly preferred are embryogenic suspensions of *Dactylis glomerata* L..

Further preferred embodiments of the present invention consist of embryogenic cell cultures (suspension cultures and callus cultures) derived from small grain cereals, especially from those selected from the genera consisting of *Avena*, *Triticum*, *Secale* and *Hordeum*.

A further objective of the present invention is the embryogenic callus derived from graminaceous plants of the subfamily *Pooideae* from which protoplasts can be isolated, wherein the protoplasts regenerate cell walls, divide and form callus capable of being regenerated into plants and preferably into fertile plants.

Embryogenic callus derived from grasses, especially from those selected from the genera consisting of *Poa*, *Festuca*, *Lolium*, *Bromus*, *Trisetum*, *Agrostis*, *Phleum*, *Alopecurus* and *Dactylis* are preferred embodiments of the present invention. Mostly preferred is embryogenic callus of *Dactylis glomerata* L..

A further preferred embodiment of the present invention consist of embryogenic callus derived from small grain cereals, especially from those selected from the genera consisting of *Avena*, *Triticum*, *Secale* and *Hordeum*.

A further objective of the present invention are graminaceous plants, preferably fertile graminaceous plants of the subfamily *Pooideae* and propagules thereof that are regenerated from protoplasts or plant cells, which are derived from said embryogenic cell culture.

Methods for the cryopreservation of cell cultures (suspension cultures and callus cultures) of all graminaceous plants

Some plant tissues can be cryopreserved by methods known in the art which are, for example, described by Withers, (1986) and in the reference cited therein. However, these methods are not generally applicable, especially not to the cryopreservation of embryogenic cell cultures (suspension cultures and callus cultures) of graminaceous plants.

It now has surprisingly been found within the scope of the present invention that embryogenic cell cultures, including suspension cultures and callus cultures of all graminaceous plants can be preserved in a suspended state by freeze preservation (cryopreservation) at low temperatures.

This method for cryopreserving embryogenic cell cultures, including suspension cultures and callus cultures of graminaceous plants comprises:

- (a) dispersing actively growing suspension culture cells or callus in a suitable liquid culture medium,
- (b) cooling down this culture to ice temperature (about 0°C to 5°C),
- (c) mixing at about the same temperature said pre-cooled culture with a suitable cryoprotecting aqueous solution,
- (d) cooling the resultant mixture at a rate of about 0.01°C to about 20°C per minute, preferably about 0.1°C to about 5°C per minute, more preferably about 0.2°C to about 2°C per minute, most preferably 0.5°C to about 1°C per minute, to a temperature between about -20°C and about -60°C, preferably between about -35°C and about -50°C, most preferably about -38°C and about -44°C,
- (e) shock-freezing the pre-cooled mixture in liquid nitrogen or liquid air, and
- (f) storing the frozen mixture at a temperature below -100°C, preferably at the temperature of liquid nitrogen or liquid air.

This method for cryopreservation of embryogenic cell cultures (suspension cultures and callus cultures) is generally applicable for all graminaceous plants. It is understood that in this context the term graminaceous plants includes but is not limited to the *Bambusoideae* (for example, *Bamboo*), the *Andropogonideae* (for example, *Saccharum*, *Sorghum* and *Zea*), the *Arundineae* (for example, *Phragmites*), the *Oryzoideae* (for example, *Oryza*), the *Panicoideae* (for example, *Panicum*, *Pennisetum* and *Setaria*) and the *Pooideae* (for example, grasses including *Poa*, *Festuca*, *Lolium*, *Bromus*, *Trisetum*, *Agrostis*, *Phleum*, *Dactylis* and *Alopecurus*, or small grain cereals including *Avena*, *Triticum*, *Secale* and *Hordeum*).

A preferred target group within the graminaceous plants is the above characterized group of *Pooideae* plants consisting of the grasses and the small grain cereals.

Typically this inventive method is carried out as follows:

A suitable amount of actively-growing callus or of suspension culture cells (normally from 1 to 40 days, preferably 2 to 10 days after subculture) is dispersed in a suitable liquid medium. Such suitable media include but are not limited to SH-0, SH-30 or SH-45 media, OMS, KM-8p, RY-2, mannitol, sucrose, or other sugar or sugar-alcohol solutions, a solution of an amino acid (for example, L-Proline) or even water. Preferable is a medium suitable for growth of the cells, or a solution of a sugar or sugar-alcohol in water. Typically, 0.01 g to 0.1 g of callus is dispersed in every ml of liquid medium and then cooled on ice.

Suitable cryoprotectant solutions, typically are mixture of osmotically active components and DMSO in water. When they are added to the pre-cooled dispersion of step (b) they normally are also pre-cooled on ice but can also have higher temperatures up to about room temperature. The temperature of the cryopreserving solution is not critical.

Representative cryoprotectant solutions include but are not limited to solutions comprising 0.5 M to 2 M Glycerol, 0.5 M to 2 M L-Proline, 0.5 M to 4 M Dimethyl Sulfoxide (DMSO) in water, pH 5.6; or 0.5 M to 2 M Glycerol, 0.5 M to 2 M Sucrose and 0.5 M to 4 M DMSO in water at pH 5 to 7. Other suitable components for cryoprotectant solutions include sugars and sugar alcohols, amino acids, and polymers such as PEG. Cryoprotectant solutions containing DMSO are preferably freshly prepared before each use or may be stored frozen. Other cryoprotectant solutions may be prepared some time before use, but are preferably prepared fresh or frozen.

Cryoprotectant solution is typically added to the solution containing dispersed suspension culture cells or callus over a period of 1 second to 4 weeks, preferably, 1 second to 1 day, more preferably, 1 second to 1 hour. The cells are exposed to the cryoprotectant solution for a suitable period of time on ice, preferably 1 minute to 2 days, more

preferably 10 minutes to 6 hours, most preferably 30 minutes to 2 hours. During or after this time, aliquots are distributed into suitable sterile cryopreservation vials or any other suitable vessel and usually kept on ice.

Prior to the addition of the said aliquots the vials can be immersed at the surface of a liquid bath which is preferably at a temperature of between 0°C and 4°C. This immersion step is not absolutely necessary but in certain cases can be of advantage. The bath may consist of ethanol or any other suitable coolant. The bath is normally equipped with a stirring device to keep the coolant mixed, and is connected to an apparatus which can refrigerate the coolant at a controlled rate.

Once the vials are in the coolant, the temperature is reduced at a suitable rate. A suitable rate can be in the range of about 0.01°C to about 20°C per minute, preferably about 0.1°C to about 5°C per minute, more preferably about 0.2°C to about 2°C per minute, most preferably about 0.5°C to about 1°C per minute. When the temperature reaches a low temperature, typically in a range between about -20°C to about -60°C, preferably between about -35°C to about -50°C, most preferably between about -38°C to about -44°C, the vials are shock-frozen, for example, by plunging them into liquid nitrogen or liquid air. The optimum temperature for plunging them into liquid nitrogen or liquid air can vary with different cultures used but is generally between -20°C and -50°C and can easily be determined by a person skilled in the art. The vials are then stored in liquid nitrogen or liquid air, either in the liquid itself or in the vapor above it, at a temperature not to exceed -100°C.

With some cultures it may be helpful to maintain the temperature of the vials at some steady low temperature for a certain period of time instead of immediately plunging into liquid nitrogen as the optimum temperature is reached.

In order to recover viable cell cultures, vials containing the callus material are removed from the liquid nitrogen. The vial is thawed, typically, with rapid agitation in a warm water bath at about 10°C to 50°C, preferably 35°C to 40°C, until all the ice has melted. Surprising-

ly, it has been found within the scope of the present invention that vials of cryopreserved cells of the *Pooideae* subfamily can be thawed by leaving them in air at room temperature until all the ice has thawed. The vials may then be kept on ice for a period of few seconds to 60 minutes, more preferably 1 to 10 minutes before the callus material contained therein being placed on a suitable culture medium.

The contents of the vial are spread onto a suitable solidified culture medium. Typically, 0.5 ml of thawed culture is spread onto each 10 cm diameter petri plate containing 30 ml to 50 ml of medium. The solid medium may be poured on a slant or a cavity may be scooped out of the medium around its periphery in order to aid the drainage of remaining cryoprotectant away from the cells. The cells may be washed one or more times with liquid culture medium, or other suitable solution, for example, a sugar or sugar alcohol solution, or a solution of an amino acid before being placed on a suitable culture medium.

The petri dishes are incubated in the dark at 27°C, as described for embryogenic callus above. Callus is then subcultured as for normal embryogenic callus as described above.

Step B: Isolation and purification of protoplasts capable of being regenerated into plants including fertile plants

Protoplasts are prepared from the embryogenic suspension cultures resulting from step A above. The isolation and purification is carried out by isolating embryogenic cell clusters from the suspension culture medium, for example, by filtering the suspension culture of step A on a Nalgene® 0.2 µm filter unit, and incubating the resultant cell clusters with a suitable enzyme preparation capable of removing the cell walls without harming the protoplasts. The enzyme is used as a filter-sterilized solution. All culture manipulations are carried out under sterile conditions using sterile materials. A suitable enzyme preparation may consist, for example, of about 2 % (w/v) of cellulase RS in 7 mM CaCl<sub>2</sub>.H<sub>2</sub>O, 0.7 mM NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, 3 mM MES [pH 5-7] and glucose (to 550 mOs/kg H<sub>2</sub>O). Usually the mixture is gently shaken on an orbital shaker at about 50 rpm in dim light (about 5 µE/m<sup>2</sup>s) but this is not



critical. The digestion is continued between 0°C and 50°C, preferably between 10°C and 35°C, most preferably between 26°C and 32°C, and until protoplasts are released. The time required for digestion is typically between several seconds and 2 days, preferably between 1 hour and 1 day, most preferably between 3 and 5 hour.

The protoplasts released are collected and cleaned by standard methods such as filtration, centrifugation and washing.

At this stage a flotation step may be included. In this case the washed protoplasts are layered on top of a suitable medium, such as KM-8p culture medium made 700 mOs/kg H<sub>2</sub>O with sucrose, or of other suitable media as described in George *et al*, (1987).

After centrifugation for about 10 minutes at about 60 g protoplasts banding at the interface are collected. Finally the protoplasts can be resuspended in the same culture medium and filtered, for example, by passing them through a stainless steel mesh screen (20 µm mesh size).

Contamination with whole undigested cells of the protoplast preparation not floated on sucrose is about 0.001 to 0.01 % of the total. With the sucrose flotation step included the cell contamination is minimal. However, the sucrose flotation step results in a significant loss of protoplasts with the densest cytoplasm. Consequently, plating efficiencies can decrease up to 10-fold when the flotation step is included. The protoplasts can also be purified using other methods known in the art, such as flotation on a sucrose solution, or other suitable buffered high density media, such as Percoll®.

Protoplasts yields and subsequent plating efficiencies are optimal if the suspension cultures used for protoplast isolation are subcultured 1 to 30 days, preferably 5 to 10 days, previously.

The enzyme mixture characterized above is a modification of that described by Lu *et al*, (1981) and is found to be superior to other mixtures tested, giving yields of 40 x 10<sup>6</sup> to 70 x 10<sup>6</sup> protoplasts per gram fresh weight. Alternatively, however, 2 % (w/v) of cellulase RS in

KM-8p culture medium or other suitable medium such as those described in Geroge *et al*, (1987) also gives respectable yields of protoplasts. Glucose is clearly superior to sucrose and somewhat superior to mannitol as the osmoticum used during the isolation of protoplasts with regard to yield and subsequent plating efficiency. Other suitable enzyme mixtures known in the art may also be used in the inventive process.

The protoplasts obtained after filtration, for example, after passage through a 20  $\mu$ m screen average 12  $\mu$ m to 15  $\mu$ m in diameter and are densely cytoplasmic.

Hence, the present invention provides a method of producing protoplasts of graminaceous plants of the subfamily *Pooideae*, which protoplasts are capable of being regenerated into plants, preferably into fertile plants. This method comprises:

- (a) isolating tissue from suitable parts of graminaceous plants of the subfamily *Pooideae*, preferably the basal parts of young inner leaves, immature sexual embryos, immature inflorescences, mature seeds or seedling tissue, most preferably the youngest inner leaves,
- (b) culturing this tissue in a medium capable of inducing the formation of embryogenic callus and of embryos,
- (c) periodically subculturing the embryogenic callus and embryos on fresh medium capable of sustaining continuous proliferation,
- (d) isolating embryogenic cell clusters after 0 to 500 transfers, preferably after 0 to 100 transfers, most preferably after 6 to 8 transfers, and
- (e) removing the cell walls with suitable enzyme mixtures and isolating and purifying the resultant protoplasts.

A further embodiment of the invention embraces protoplasts (including the plant cells after regeneration of the cell walls) of graminaceous plants of the subfamily *Pooideae* capable of being regenerated into plants, especially fertile plants. Preferred are protoplasts or cells either derived from cell cultures or from embryogenic cell suspensions.

Also comprised are graminaceous plants, preferably fertile graminaceous plants, of the subfamily Pooideae and propagules thereof regenerated from said protoplasts or plant cells.

Step C. Establishment of protoplast cultures and growth of callus capable of being regenerated into plants and fertile plants.

The purified protoplasts of step B) above are plated with or without treatment with exogenous DNA in a suitable liquid or a solidified medium (The treatment with exogenous DNA will be described in detail in a subsequent paragraph). Some suitable media include those based on KM-8p; RY-2 [Potrykus et al, 1979]; and SH-30 and SH-45, with appropriate concentrations of sugars and plant growth regulators. The preferred solidifying agent is agarose, especially SeaPlaque<sup>®</sup> agarose [FMC Corp. Marine Colloids Division, P.O. Box 308, Rockland ME 04841, USA]. Where used, the concentration of SeaPlaque<sup>®</sup> agarose may be between 0.1 % and 2.5 % (w/v), preferably between 0.6 % and 1.5 % (w/v).

The plating of protoplasts on an agarose-containing medium can be carried out in accordance with the methods described by Shillito et al, (1983); or in the European Patent Application EP-0,129,688 (Shillito et al) published 2.1. 1985; or by Adams et al, (1983).

The medium in which the protoplasts are cultured may contain suitable substances to assist the protoplasts to divide and form colonies. These substances include, for example, 2,4-D, dicamba, picloram, or other plant growth regulators. Suitable plant growth regulators are known in the art. The concentration of such substances is usually in the range of 0.01 mg/liter to

100mg/liter.

Salicylic acid and its derivatives may promote division of, and/or colony formation from, Pooideae protoplasts. The derivatives of salicylic acid include but are not limited to O-acyl and O-aryl derivatives. The O-acyl derivatives include but are not limited to the short chain acyl groups, such as those having 1 to 7, preferably 1 to 4, and most preferably 2 to 3 carbon atoms. The O-aryl derivatives include but are not limited to those

having one or more 5 or 6 membered rings, which may be fused or unfused. The rings may be unsubstituted or substituted with one or more groups including alkyl having 1 to 5 carbon atoms, O-alkyl having 1 to 4 carbon atoms, halogen (especially chlorine and bromine), nitro; amino, and amino substituted by alkyl having 1 to 4 carbon atoms.

The derivatives of salicylic acid also include the carboxylate esters. The preferred carboxylate esters are the aryl, and alkyl esters wherein the alkyl group has 1 to 4 carbon atoms.

The derivatives of salicylic acid, in addition, include compounds wherein the salicylic acid ring is further substituted by, for example, one or more groups including alkyl having 1 to 4 carbon atoms, O-alkyl having 1 to 4 carbon atoms, halogen (especially chlorine and bromine), nitro; amino, and amino substituted by alkyl having 1 to 4 carbon atoms.

Preferred compounds promoting the division of, and/or the colony formation from, *Pooideae* protoplasts and cells are:

O-acetoxybenzoic acid (aspirin, acetylsalicylic acid);

O-hydroxybenzoic acid (salicylic acid);

O-methoxybenzoic acid (methylsalicylic acid); and

O-dimethylcarbamoylbenzoic acid [O-(CO-dimethyl)-salicylic acid].

The concentration of the salicylic acid or a derivative thereof in the culture medium is suitably in the range of 0.1 mg/liter to 3'000 mg/liter, preferably in the range of 10 mg/liter to 300 mg/liter, and most preferably about 100 mg/liter.

The medium in which the protoplasts are cultured may contain medium which has previously been conditioned by the growth of suitable cells such as, for example, *Zea mays*, *Dactylis glomerata* or other *Gramineae*, or even other (dicotyledoneous) plants. Preferred is medium in which an embryogenic suspension of graminaceous species has been grown.

Most preferred is medium in which embryogenic suspension of *Dactylis glomerata* has been grown. The medium conditioned as described above may be in the proportion of between 0 % and 100 % (v/v), preferably between 5 % and 50 % (v/v), more preferably between 30 % and 40 % (v/v) of the total medium.

The protoplasts may be cultured in the solid or liquid medium without subculture for a period of up to 12 weeks, preferably up to 6 weeks, most preferably in the range of 1 to 3 weeks. In a preferred embodiment of the present invention a solid medium can be placed in a liquid medium, as described in EP-0,129,688 (Shillito *et al.*) or treated in some other manner to assist division of, and/or colony formation from the protoplasts.

The protoplasts are cultured in the light or, preferably in the dark at a temperature range between 0°C and 50°C, preferably between 20°C and 32°C, most preferably between 25°C and 28°C. The light intensity is typically between 0.1  $\mu\text{E}/\text{m}^2\text{s}$  and 200  $\mu\text{E}/\text{m}^2\text{s}$ , preferably between 30  $\mu\text{E}/\text{m}^2\text{s}$  and 90  $\mu\text{E}/\text{m}^2\text{s}$ .

Plating efficiencies obtained using KM-8p medium vary from 0.5 % to 10 % depending on the quality of the protoplast preparation. The addition of 30 % to 40 % (v/v) conditioned suspension culture medium (suspension culture medium conditioned by growth of cells therein and made up to 550 mOsm/kg H<sub>2</sub>O by addition of glucose) to the protoplast culture medium does not result in a significant increase in plating efficiency but does accelerate the division process in the young protoplast-derived colonies.

In a preferred embodiment of the present invention the protoplasts are plated in an agarose solidified medium. The first cell divisions are noticed about two days after plating of the protoplasts. Subsequent divisions occur every 2 to 3 days. The process is not synchronous, as shown by the fact that first divisions can still be observed after 7 days. After 5 to 20 days, preferably after 10 to 14 days, after plating, the agarose solidified medium is cut into segments and the segments containing the cell colonies are transferred to a liquid

nutrient medium. This procedure is known in the art as 'bead culture technique' and is fully described by Shillito *et al*, (1983) and in EP-O, 129, 688 (Shillito *et al*).

Instead of cutting the agarose solidified medium it is also possible to liquefy the agarose medium and to transfer the liquefied medium to the liquid nutrient medium. This modification can be carried out in accordance with Adams *et al*, (1983). In both cases (cutting or liquifaction) the liquid component can remain KM-8p medium with glucose or sucrose with good colony growth observed. However, the optimal liquid component with regard to the growth rate is SH-45 medium with 4 g/liter casein hydrolysate. Within about 2 to 3 weeks of initiating the bead cultures, new suspension cultures can be observed in the plates. Microscopic examination of the agarose slabs reveals that normally some of the colonies closest to the surface grow out into the liquid and release small masses of cells while still anchored in the agarose. The new suspensions multiply rapidly and after another two weeks are transferred as suspension cultures in the usual manner or plated onto SH-30 plates for callus development. Alternatively the agarose can be spread on plates containing agarose solidified SH-45 medium, and colonies allowed to grow out.

Hence, the present invention also relates to a method of producing cell cultures (suspension cultures or callus cultures) from protoplasts of graminaceous plants of the subfamily *Pooideae*, which cell culture is capable of being regenerated into plants, including fertile plants. This process comprises:

- (a) culturing in a suitable culture medium protoplasts of graminaceous plants of the subfamily *Pooideae* capable of being regenerated into plants until they form cell colonies, and
- (b) culturing said cell colonies or parts thereof on a medium suitable to promote cell culture formation, and
- (c) isolating the resultant cell culture.

Step (b) is not absolutely necessary. It is also possible to let the protoplasts stay in the medium step (a) until cell culture or embryos are formed.

Also comprised are graminaceous plants, especially fertile graminaceous plants, of the subfamily *Pooideae* regenerated from said cell cultures.

A preferred embodiment of the present invention comprises plating the protoplasts on a agarose solidified medium, liquefaction or segmentation of the agarose solidified medium, transferring the liquefied or segmented medium to a liquid nutrient medium, and culturing until cell colonies are formed.

Preferred is a method wherein the parts of the cell colonies mentioned above under step (b) arise from cells and/or cell masses released to the liquid nutrient medium.

The callus and suspension cultures produced in this and other steps may be cryopreserved as described in step A.

#### Step D: Regeneration of plantlets from callus

Callus derived from protoplasts [step C], preferably friable granular callus, is subcultured one or more times, preferably every two weeks, onto a suitable fresh medium so as to induce embryo formation. Suitable inducing media include a SH medium with appropriate concentrations of sugars and plant growth regulators.

Any embryos which are formed are removed and plated on a medium suitable to induce them to mature and germinate. Suitable media include, for example, SH-30 or OMS medium containing modifications to contain the appropriate amounts of sugars and plant growth regulators. The plates are placed in the light [10  $\mu\text{E}/\text{m}^2\text{s}$  to 200  $\mu\text{E}/\text{m}^2\text{s}$  from cool white fluorescent lamps or from a mixture of daylight and Gro-lux® (Sylvania) fluorescent lamps or any other suitable fluorescent lamp]. Mature embryos are observed about 2 to 5 weeks after plating. In some cases one or more extra transfers to fresh medium can be beneficial for completing embryo maturation. The embryos differentiate further to form plantlets after a suitable period of time, typically 1 week to 6 months, more typically 1 to 3 months.



Alternatively, callus derived from protoplasts [step C], preferably friable granular callus, is subcultured one or more times, preferably every two weeks, onto a suitable fresh medium so as to induce embryo formation and maturation. Suitable media include but are not limited to OMS medium with appropriate concentrations of sugars and without plant growth regulators. The plates are placed in the light [10  $\mu\text{E}/\text{m}^2\text{s}$  to 200  $\mu\text{E}/\text{m}^2\text{s}$  from cool white fluorescent lamps or from a mixture of daylight and Gro-lux® (Sylvania) fluorescent lamps or any other suitable fluorescent lamp]. The embryos differentiate further to form plantlets after a suitable period of time, typically 1 week to 6 months, more typically 1 to 3 months.

Step E: Obtaining plants, preferably fertile plants, from plantlets

Plantlets obtained in accordance with step D above are transferred to a suitable medium such as for example SH-0 or OMS, which contain no growth regulators. Alternatively, a growth regulator stimulating root or shoot growth may be added. Suitable growth regulators are known in the art. The plantlets are cultured on said medium until they form roots. It is important to remove all callus from the plantlets, since this newly formed callus is found to be inhibitory to the growth of the plantlets. To this end the plantlets can be washed with sterile distilled water upon transfer. Callus which arises subsequently must be removed at regular intervals, preferably every 3 to 30 days, more preferably every 1 to 2 weeks. The time required for root formation is typically about 1 to 4 weeks, more typically about 2 weeks. Plantlets with a good root system can be transferred to soil in the greenhouse and hardened off gradually. A sufficient length for the roots at this stage is the range of 1 cm to 10 cm, more typically 2 cm to 5 cm, and for the shoot is the range of 1 cm to 10 cm, more typically 2 cm to 5 cm. Alternatively, the plantlets can be subcultured indefinitely in-vitro by separation of the tillers and placing the plantlets on fresh medium such as SH-0 or OMS.

Thus, the inventive method of regenerating graminaceous plants, preferably fertile plants, of the subfamily *Pooideae* from callus comprises

- (a) culturing callus of *Pooideae* plants, which callus is derived from protoplasts and is capable of being regenerated into plants on a medium capable of inducing embryo formation until embryos are formed,
- (b) culturing the embryos on a medium suitable to induce them to mature and germinate, and
- (c) culturing the resultant plantlets until sufficiently developed to be transferred to soil to form mature plants.

Flowering can be induced as described by Heide, (1987) or as appropriate for the particular species or variety being used. Methods for inducing flowering are known in the *Pooideae*. Seed produced from these plants can be treated in an appropriate way to induce germination, and sown either in pots or sterilized and plated on Murashige and Skoog medium without growth regulators (OMS medium) and solidified with 0.8 % agar or agarose, or GelRite® or any other suitable gelling agent. The seed can also be sown on medium containing between 10 µg/ml and 1'000 µg/ml hygromycin B to determine the inheritance of the hygromycin resistance character.

Thus, the inventive method of regenerating fertile graminaceous plants of the subfamily *Pooideae* from callus comprises

- (a) culturing callus of *Pooideae* plants, which callus is derived from protoplasts and is capable of being regenerated into fertile plants on a medium capable of inducing embryo formation until embryos are formed,
- (b) culturing the embryos on a medium suitable to induce them to mature and germinate,
- (c) culturing the resultant plantlets until sufficiently developed to be transferred to soil to form mature plants, and
- (d) obtaining seed following controlled or open pollination.

A further embodiment of the present invention are graminaceous plants, especially fertile graminaceous plants of the subfamily *Pooideae* regenerated from said callus.

Step F: Treating the protoplasts with exogenous DNA

Pooideae protoplasts may be treated with exogenous DNA so as to produce cells that contain all or part of the exogenous DNA stably integrated into their genetic material. Exogenous DNA should be understood within this invention as comprising any DNA added to a protoplast. It may be homologous or heterologous to the plant being transformed. The exogenous DNA may contain a promoter active in graminaceous plants, preferably in plants of the subfamily Pooideae, or may utilize a promoter already present in the plant genome. The exogenous DNA may contain one or more genes which alter the genotype and especially the phenotype of the resultant cells or of the plants being regenerated from transformed cells. It is desired, however, that the genetic sequence encoding one or more desired proteinaceous products be expressed, and produces one or more functional enzymes or polypeptides in the resulting cell and plant respectively. The exogenous DNA may be a chimeric gene, or a portion thereof.

Within the scope of the present invention exogenous DNA should also be understood as to comprise non-coding regulatory DNA sequences, that are, for example, involved in the regulation of the transcription process.

Treatment of the protoplasts with exogenous DNA can be carried out by methods such as those described in the following publications [Paszkowski et al, 1984; European Patent Application EP-0,164,575, (Paszkowski et al) published 18.12.1985; Shillito et al, 1985; Potrykus et al, 1985; Loerz et al, 1985; Fromm et al, 1986; British Patent Application GB-2,140,822 (Mettler) published 5.12.1984; and Negrutiu et al, 1987].

The exogenous DNA may be added in any form such as, for example, naked linear or circular DNA, DNA encapsulated in liposomes, DNA in sphaeroplasts, DNA in other plant protoplasts, DNA complexed with salts, etc. Uptake of foreign DNA may be stimulated by any suitable method known in the art including the methods described in the references quoted above.

Primarily, the chimeric genes contemplated in this invention are those which provide the transformed plant protoplasts, protoplast-derived tissues and finally the protoplast-derived plants with valuable properties, such as increased resistance to pathogens (e.g. to phytopathogenic fungi, bacteria, viruses, etc.); resistance to chemicals [e.g. to herbicides (such as triazines, sulfonylureas, imidazolinones, triazolo-pyrimidines, bialaphos, glyphosate, etc.), insecticides or other biocides]; resistance to cytotoxins (e.g. to hygromycin, kanamycin, chloramphenicol, etc.); resistance to adverse environmental (edaphic or atmospheric) influences (e.g. to heat, cold, wind, unfavorable soil conditions, moisture, dryness, etc.); or with increased formation of reserve or storage substances in the leaves, seeds, tubers, roots, stalks, etc. Desirable substances produced by a transgenic plant include, for examples, proteins, starches, sugars, amino acids, alkaloids, flavors, colors, fats, etc.

Resistance to cytotoxins may, for example, be conferred by a gene expressing in the plant cells an enzyme that detoxifies the cytotoxin, for example, neomycin phosphotransferase type II or aminoglycoside phosphotransferase type IV for detoxification of kanamycin, hygromycin and other aminoglycoside antibiotics, or a glutathione-S-transferase or cytochrome P-450 or other catabolic enzyme known to detoxify triazine, sulfonylurea or other herbicides. Resistance to cytotoxins may also be conferred by a gene that expresses in a plant a form of a "target enzyme" (site of the action of the cytotoxin) which is resistant to the cytotoxin, for example, a form of aceto hydroxyacid synthase which is insensitive to inhibition by sulfonylureas or imidazolinones, or other herbicide acting at this metabolic step, or a form of EPSP synthase that is insensitive to inhibition by glyphosate. It can be advantageous to express these altered target enzymes in a form that allows their transport in the plant cell into the correct cellular compartment, i.e. the chloroplast in the above examples.

In certain cases it is advantageous to target the gene products into the mitochondria, the vacuoles, into endoplasmatic vesicles, or other cell parts or even into the intercellular (apoplastic) spaces.

Resistance to certain classes of fungi may be conferred, for example, by the introduction of a gene that expresses chitinase in the plant tissues. Many plant pathogenic fungi contain chitin as an integral part of hyphal and spore structure, e.g. basidiomycetes (smuts and rusts) and ascomycetes and *fungi imperfecti* (including *Alternaria* and *Bipolaris*, *Exerophilum turcicum*, *Colletotricum*, *Gleocercospora* and *Cercospora*). Chitinase can inhibit the growth of mycelia of certain pathogens *in vitro*. A plant leaf or root expressing chitinase constitutively or in response to a pathogen invasion is protected against many types of fungal attack. Constitutive expression may or may not be advantageous over the inducible expression that is normal response to pathogen attack in certain plants, because the chitinase is present immediately at high level with no lag time required for de novo synthesis.

Insect resistance may, for example, be conferred by a gene encoding a polypeptide that is toxic to insects and/or their larvae, such as the crystalline protein of *Bacillus thuringensis* [Barton *et al*, 1987; Vaeck *et al*, 1987]. A second class of protein which will confer insect resistance are protease inhibitors. Protease inhibitors are common constituents of plant storage structures [Ryan, 1973]. Purified Bowman-Birk protease inhibitor isolated from soybean has been shown to inhibit gut protease of *Tenebrio* larvae [Birk *et al*, 1963]. The gene encoding cowpea trypsin inhibitor is described by Hilder *et al*, (1987).

A gene encoding a protease inhibitor may be placed under the control of a plant promoter, preferably a constitutive promoter such as CaMV 35S promoter [which is described by Odell *et al*, (1985)], in a suitable vector. The gene, for example, the coding sequence for the soybean Bowman-Birk protease inhibitor, may be obtained using the cDNA cloning methods described by Hammond *et al*, (1984). An alternative method of obtaining a gene for protease inhibitors with less than 100 amino acids, such as the lima bean trypsin inhibitor, is to synthesize it. The coding sequence is predicted by back-translation and restriction sites appropriate for the desired vector are included at each end. The synthetic gene is prepared by synthesizing overlapping oligonucleotides of 30 to 60 bases. The fragments are kinased, ligated (Maniatis *et al*, 1982) and cloned into the appropriate vector. A clone whose insert is in the

correct orientation may be identified by sequencing. Plasmid DNA is isolated and used for incorporation into the protoplasts (Abel *et al.*, 1986).

Also included in the present invention are genes coding for pharmaceutically active ingredients, for example, alkaloids, steroids, hormones and other physiologically active substances, and flavins, vitamins and colorings. Therefore, genes which are contemplated in this invention include, but are not limited to, plant specific genes, such as the zein gene (Wienand *et al.*, 1981) mammalian specific genes, such as the insulin gene, the somatostatin gene, the interleukin gene, the t-PA-genes (Pennica *et al.*, 1983) etc., or genes of microbial origin, such as the NPT II gene as well as genes of synthetic origin such as the insulin gene (Itakura *et al.*, 1975).

As a matter of course, also non-coding DNA sequences that are, for example, involved in the control of transcription process, can be used for the transformation of plant protoplasts of plants of the subfamily *Pooideae*.

Several plant genes are known to be induced by various internal and external factors including plant hormones, heat shock, chemicals, pathogens, lack of oxygen and light.

As an example of gene regulation by a plant hormone, abscisic acid (ABA) has been shown to induce the late embryogenesis abundant mRNAs of cotton.

In another example, gibberellic acid (GA3) induces malate synthase transcripts in castor bean seeds and alpha-amylase isozymes in barley aleurone layers.

The regulation of heat shock protein genes of soybean has been studied in detail. Treatment of plants for several hours at 40°C results in the de novo synthesis of several so-called heat shock proteins. Several such genes have been isolated and their regulation studied in detail. The expression of these genes is primarily controlled at the level of transcription (Shoffl *et al.*, cited in Willmitzer 1988). The promoter of the

hsp70 gene has been fused to the neomycin phosphotransferase II (NptII) gene and the chimeric gene has been shown to be induced by heat shock (Spena *et al*, 1985).

Another class of inducible genes in plants include the light regulated genes, most notably the nuclear encoded gene for the small subunit of ribulose 1,5-bisphosphate carboxylase (RUBISCO). Morelli *et al*, (1985) and Mererria-Estrella *et al*, (1984) have demonstrated that the 5'-flanking sequences of a pea RUBISCO gene can confer light inducibility to a reporter gene when attached in a chimeric fashion. This observation has been extended to other light inducible genes such as the chlorophyll a/b binding protein.

The alcohol dehydrogenase (adh) genes of maize have been extensively studied. The adh1-s gene from maize was isolated and a portion of the 5'-flanking DNA was shown to be capable of inducing the expression of a chimeric reporter gene (e.g., chloramphenicol acetyl transferase, CAT) when the transiently transformed tissue was subjected to anaerobic conditions (Howard *et al*, 1987).

In a preferred embodiment of the present invention *Pooideae* protoplasts are transformed by means of a combination of electroporation and polyethylene glycol treatment. Immediately after the purification of the protoplasts obtained in step B, electroporation is performed as described by Shillito *et al* (1985) or in EP-0,164,575 (Paszkowski *et al*). The protoplasts are resuspended in an electroporation buffer after the last wash. Suitable electroporation buffers include aqueous solutions of mannitol containing an appropriate amount of  $MgCl_2$ . An aqueous solution of DNA is added to the protoplast suspension. In one embodiment the DNA is plasmid pCIB709 linearized by treatment with a suitable restriction endonuclease. The resulting mixture is gently mixed. In one embodiment, one half volume of a 24 % (w/v) solution of PEG in 0.5 M mannitol and 30 mM  $MgCl_2$  is added. After mixing the protoplasts are transferred to the chamber of a Dialog® Electroporator [DIA-LOG G.m.b.H., Haffstrasse 34, D-4000 Düsseldorf 13, FRG], and 2 to 5 pulses, preferably 3 pulses of about 2'000 V/cm to 5'000 V/cm initial voltage and exponential decay constant of 10  $\mu$ sec are applied at 30 sec intervals. The sample is then



placed in a petri plate and 1 % (w/v) to 25 % (w/v) of agarose as solidifying agent is added, the protoplasts distributed throughout the medium, and the agarose allowed to set. From this culture of transformed protoplasts transgenic *Pooideae* plants, including fertile transgenic *Pooideae* plants, are regenerated as described under steps C to F above.

In a further preferred embodiment of the present invention, *Pooideae* protoplasts are transformed according to the method described by Negrutiu *et al.*, (1987). In this case the purified protoplasts are suspended following the last wash in 0.5 M mannitol containing between 15 mM and 45 mM MgCl<sub>2</sub>. DNA is added in an aqueous solution, and then an equal volume of a 36 % (w/v) solution of PEG is added [Negrutiu *et al.*, 1987]. The resulting mixture is gently mixed and incubated for 5 to 60 minutes, preferably for about 30 minutes, at temperatures between 10°C and 32°C, preferably at room temperature (about 25°C). During incubation the mixture is occasionally shaken. After incubation the protoplasts are washed and plated on a suitable culture medium. Suitable culture media include but are not limited to KM-8p medium containing 0.3 % (w/v) to 2.5 % (w/v) agarose, preferably 0.6 % (w/v) to 2 % (w/v) agarose as a solidifying agent. The transformed protoplasts are distributed throughout the medium, and the agarose is allowed to gel. From this culture, transgenic, *Pooideae* plants, including fertile *Pooideae* plants are regenerated in accordance with steps C to E above.

A preferred exogenous DNA within the scope of this invention is the plasmid pCIB709 as shown in figure 7, in its linearized form.

#### Step G: Selection of transformed colonies

The agarose solidified medium [step F] containing the transformed protoplasts is incubated in the light or preferably in the dark for 5 to 30 days, preferably 8 to 15 days, more preferably for 10 days, at a temperature range between 0°C and 50°C, preferably 20°C and 32°C, more preferably 25°C and 28°C. The solidified medium is cut into, for example, 5 slices and selected in the 'bead type' culture system in accordance with the methods described by Shillito *et al.*, (1983); or in the European Patent Application EP-0,129,688 (Shillito *et al.*); or in Shillito *et al.*,

(1985); or in European Patent Application EP-0,164,575 (Paszkowski et al). Number and size of the slices are not critical. In one embodiment, four of these slices are put separately into a suitable medium, such as, for example, SH-45 culture medium containing 4 g/liter casein hydrolysate and 20 µg/ml to 100 µg/ml hygromycin B. The fifth slice is put into the same medium but without hygromycin B (control).

After about 4 to 5 weeks the putative transformed cell colonies are cut out of the agarose and cultivated in suitable culture medium, such as SH-45 containing 20 µg/ml to 100 µg/ml hygromycin B, which is agitated at, for example, 50 rpm to 80 rpm on an orbital shaker. After another 4 to 5 weeks all colonies which make a new suspension culture are transferred to a new medium containing 20 µg/ml hygromycin B. The new suspensions are grown for a minimum of two subcultures in the presence of 20 µg/ml hygromycin B and incubated under the same conditions as described above until callus is formed.

Callus and suspension cultures, and cultures derived from the materials produced in this step may be cryopreserved as described in step A.

Step H: Regeneration of transformed *Pooideae* plants from callus

Transformed *Pooideae* plants are regenerated from transgenic callus of step G in accordance with the procedure described under steps D to E above.

The method of the present invention permits protoplasts of graminaceous plants of the subfamily *Pooideae* to be regenerated into plants, and more preferably, into fertile plants. This possibility enables one for the first time to introduce exogenous DNA stably into the genome of such plants, and to alter their geno- and phenotypes. In addition the protoplasts can be fused with protoplasts from the same or another species, in order to produce novel combination of nuclear DNA, or novel combinations of nuclear and organelle DNA. Moreover, the protoplasts can be used as a source of clonal material, on which mutagenesis and/or selection for a desired phenotype can be carried out.

1341473

Examples of desired phenotypes include resistance to toxic concentrations of natural or synthetic chemicals including but not limited to insecticides, herbicides, fungicides, bactericides, heavy metals, salts, pathotoxins, metabolic inhibitors, structural or functional analogs of cellular metabolites. Other examples of desirable phenotypes which can be selected for include resistance to adverse environmental conditions such as cold or warm temperatures or to biotic agents such as pathogens.

The following experiments and examples further illustrate the present invention in detail but are not be construed to limit the scope thereof.

#### Nonlimiting Examples

##### Example 1: Preparation of embryogenic suspensions from tissue of *Dactylis glomerata* L. (orchardgrass)

Embryogenic callus is initiated from basal sections of the youngest leaves of greenhouse-grown orchardgrass plants (*Dactylis glomerata* L.) as described by Hanning et al, (1982). The leaves are surface sterilized by immersion in a 1:10 dilution of Clorox<sup>®</sup> solution [a solution of 5.25 % (w/v) Sodium hypochlorite; The Clorox Company, Oakland, CA94623, USA] for about 10 minutes and then cut aseptically into small segments of 1 mm to 5 mm in length or in diameter. These segments are plated on sterile SH-30 medium containing 0.8 % (w/v) agarose as a gelling agent. Callus and/or embryogenic structures appear within 2 to 6 weeks after plating, upon culture at about 25°C. Embryogenic callus was maintained by subculturing onto fresh SH-30 medium every 2 to 4 weeks and culturing in the dark at 25°C.

Embryogenic suspension cultures are initiated by placing approximately 0.5 g fresh weight of embryogenic callus into 50 ml of liquid medium described by Gray, et al, (1985) containing 45 µM dicamba and 4 g/liter casein hydrolysate. The suspension cultures are grown at 27°C under a 16 hours light (40 µE/m<sup>2</sup>s), 8 hours dark photoperiod on a gyratory shaker at about 130 rpm in 125 ml Delong flasks sealed with a metal cap and parafilm<sup>®</sup>. After approximately four weeks the large cellular clumps are allowed to settle for about 30 seconds and 10 ml aliquots of the supernatant medium containing small cell clusters are removed and transferred

\*Trade-mark

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to 50 ml of fresh medium. This process is repeated every 3 to 4 weeks using the most successful cultures as judged by smaller clump size and better quality based on the presence of small, cytoplasmic cells. After 5 to 8 transfers the suspensions are essentially free of non embryogenic cells and the majority of the embryogenic cell clusters are quite small (150  $\mu$ m to 2'000  $\mu$ m).

Example 2: Isolation and purification of *Dactylis glomerata* L.

protoplasts

Protoplasts are prepared from embryogenic suspension cultures of example 1 by aseptically filtering the cells on a Nalgene® 0.2  $\mu$ m filter unit and then adding 0.5 g fresh weight cells to each 12.5 ml of protoplast enzyme mixture in a petri dish. The enzyme mixture consists of 2 % (w/v) Cellulase RS; 7 mM  $\text{CaCl}_2 \times \text{H}_2\text{O}$ ; 0.7 mM  $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$ ; 3 mM MES (pH 5.6); glucose (550 mOs/kg  $\text{H}_2\text{O}$  of pH 5.6); and is filter sterilized. The mixture is swirled on an orbital shaker at about 50 rpm in dim ( $< 5 \mu\text{E}/\text{m}^2\text{s}$ ) light for about 4 to 5 hours. The digest is then sieved through a stainless steel sieve (100  $\mu$ m mesh size) and distributed into 12 ml centrifuge tubes which are centrifuged at about 60 g to 100 g for about 5 minutes. The protoplast-containing sediment is then washed three times with protoplast culture medium KM-8p adjusted to 550 mOs/kg  $\text{H}_2\text{O}$  with glucose. At this point a flotation step may be included for further purification of the protoplasts. In this case, the washed protoplasts are layered atop 10 ml of KM-8p culture medium adjusted 700 mOs/kg  $\text{H}_2\text{O}$  with sucrose. After centrifugation at 60 g to 100 g for about 10 minutes, protoplasts banding at the interface are collected using a fine pipette. Finally, the protoplasts are resuspended in 1 ml to 2 ml KM-8p culture medium and sieved through a stainless mesh screen (20  $\mu$ m mesh size). The protoplasts released are collected and washed and resuspended in KM-8p medium for culture or in osmotically adjusted medium suitable for transformation according to Example 6.

Example 3: *Dactylis glomerata* L. protoplast culture and growth of

callus

- (a) The purified protoplasts are plated at a density of about  $5 \times 10^5$  protoplasts/ml in KM-8p culture medium containing 1.3 % (w/v) SeaPlaque® agarose [FMC Corp., Marine Colloids Division, Rockland,

1341473

Maine, USA] and 30 % to 40 % (v/v) of conditioned medium (obtained from 3 to 4 week-old *Dactylis glomerata* L. embryogenic suspension cultures by filtering the medium through a sterile Nalgene® 0.2 µm filter, making the medium 550 mOsm/kg H<sub>2</sub>O by addition of glucose, and again filter sterilizing). The plates are then placed in the dark at a constant temperature of 28°C. After 10 to 14 days the agarose is cut into wedges and placed into "bead culture" as described by Shillito *et al*, (1983) using 20 ml SH-45 suspension culture medium with 3 % (w/v) sucrose per 3 ml original agarose embedded culture. The plates are put on a platform shaker and agitated at about 50 rpm in light at 8 µE/m<sup>2</sup>s. New suspension cultures are formed as the colonies grow out of the agarose and release cells into the liquid medium. The resultant suspension cultured cells are plated onto agar-solidified SH-30 medium and placed in the dark at 25°C until callus is formed.

- (b) Protoplasts are cultured as described in example 3(a) above except that the culture media contains an addition of 100 mg/liter O-acetyl-salicylic acid.
- (c) Protoplasts are cultured as described in example 3(a) above except that the culture media contains an addition of 30 mg/liter O-acetyl-salicylic acid.
- (d) Protoplasts are cultured as described in example 3(a) to 3(c) above except that the culture media contains no conditioned medium.

Example 4: Regeneration of *Dactylis glomerata* L. plants from protoplast-derived callus

- a) *Dactylis glomerata* L. callus (obtained as described in Example 3) derived from protoplasts is grown on solidified SH-30 medium, and subcultured every two weeks. Any embryos which form are removed and plated on germination medium (SH-0) and placed in the light (45 µE/m<sup>2</sup>s to 55 µE/m<sup>2</sup>s). Germination of these embryos occurs in 1 to 4 weeks and the resultant plantlets are placed on SH-0 medium in the light to form root systems. They are moved into the greenhouse at the six to twelve leaf stage, and hardened off gradually.

- b) Callus (obtained as described in Example 3) derived from protoplasts is grown on SH-O medium solidified with 0.24 % (w/v) GelRite® in the light (45  $\mu\text{E}/\text{m}^2\text{s}$  to 55  $\mu\text{E}/\text{m}^2\text{s}$ ), and subcultured every two weeks. The resultant plantlets are placed on a 1:1 mixture of SH-O and OMS media solidified with a combination of 0.12 % (w/v) GelRite® and 0.4 % (w/v) agar in the light to form root systems. They are moved to the greenhouse at the six to twelve leaf stage, and hardened off gradually.
- c) Small plantlets are obtained as described in 4(a) and 4(b) above, and are placed on OMS medium solidified with 0.8 % (w/v) agar in the light to form root systems. They are moved to the greenhouse at the six to twelve leaf stage, and hardened off gradually.
- d) Small plantlets are obtained as described in 4(a) above and are placed on a 1:1 mixture of SH-O and OMS media solidified with a combination of 0.12 % (w/v) GelRite® and 0.4 % (w/v) agar in the light to form root systems. They are moved to the greenhouse at the six to twelve leaf stage, and hardened off gradually.

Example 5: Construction of plasmid pCIB709, an *E.coli* replicon bearing a plant expressible hygromycin-resistance gene [35S/Hyg<sup>r</sup>]

The coding sequence for the structural gene encoding hygromycin-resistance is isolated from the plasmid pLG90 (Gritz and Davies, 1983) on a BamHI fragment approximately 1150 bases in size. The plasmid pLG90 is available from Linda Gritz [Applied Biotechnology, 80 Rogers St., Cambridge, Mass. 02141]. This BamHI fragment is inserted into the BamHI site of pCIB710 (Rothstein et al, 1987) to construct the plasmid pCIB709. The plasmid pCIB710 contains the regulatory regions of the CaMV [cotton flower mosaic virus 35S transcript with the promoter and terminator region separated by a unique BamHI site. The resulting plasmid, pCIB709, has been deposited with ATCC, accession number 40428.

Before use in transformation, the plasmid pCIB709 can be linearized by treatment with restriction endonuclease PvuII. This construct contains a hygromycin-resistance (aminoglycoside phosphotransferase type IV) gene together with the 5' and 3' expression signals of the CaMV 35S transcript from cauliflower mosaic virus (CaMV) in a pUC plasmid. The sequence of pCIB709 is given in figure 7.

Example 6: Transformation of *Dactylis glomerata* L. protoplasts by means of electroporation

(a) Immediately after purification of the protoplasts, electroporation is performed according to Shillito et al, (1985) using the linearized plasmid pCIB709 as shown in figure 7. The protoplasts are resuspended after the last wash at a density of about  $7 \times 10^6$  protoplasts/ml in the electroporation buffer (0.4 M mannitol, 6 mM  $MgCl_2$ ). The protoplasts are placed in 0.7 ml aliquots in 10 ml plastic centrifuge tubes. Plasmid DNA (62  $\mu$ l water containing pCIB709 restricted with PvuII and sonicated calf thymus DNA [Sigma] to give final concentrations of plasmid pCIB709 and calf thymus DNA of 10  $\mu$ g/ml and 50  $\mu$ g/ml respectively) is added to the tubes. Then 0.38 ml polyethylene glycol (PEG) solution [24 % (w/v) PEG 6000 in 0.4 M mannitol, 30 mM  $MgCl_2$ , 0.1 % (w/v) MES (pH 5.6)] is added and the solution gently mixed. The protoplast suspension is transferred into the chamber of a Dialog® Electroporator and 10 pulses of 3'250 V/cm initial voltage and exponential decay constant of 10  $\mu$ sec applied at 30 sec intervals. The sample is removed from the chamber, and placed in a 10 cm diameter petri dish. 10 ml of KM-8p medium containing 1.2 % (w/v) SeaPlaque® agarose is added, the protoplasts distributed even throughout the medium, and the agarose allowed to gel.

(b) Example 6(a) is repeated except that the initial voltage used is 3'500 V/cm.

(c) Example 6(a) is repeated except that the initial voltage used is 4'000 V/cm.

- (d) Example 6(a) is repeated except that the initial voltage used is 5'000 V/cm.
- (e) Example 6(a) is repeated except that the initial voltage used is 3'000 V/cm.
- (f) Example 6(a) is repeated except that the initial voltage used is 2'500 V/cm.
- (g) Examples 6(a) to 6(f) are repeated except that PEG of MW 4'000 is used.
- (h) Example 6(a) to 6(f) are repeated except that PEG of MW 8'000 is used.
- (i) Examples 6(a) to 6(h) are repeated except that the final PEG concentration is between 10 % and 30 % (w/v).
- (j) Examples 6(a) to 6(i) are repeated except that in addition a heat shock as described in Shillito *et al*, (1985) and Potrykus *et al*, (1985) is used.

Example 7: Transformation of *Dactylis glomerata* L. protoplasts by treatment with polyethylene glycol (PEG)

- (a) PEG mediated direct gene transfer is performed according to Negrutiu *et al*, (1987). The DNA used is the linearized plasmid pCIB709.

The protoplasts are suspended following the last wash in 0.5 M mannitol containing 15 mM MgCl<sub>2</sub> at a density of about  $2 \times 10^6$  protoplasts per ml. The protoplast suspension is distributed as 1 ml aliquots into 10 ml plastic centrifuge tubes. The DNA is added as described in example 6 above, and then 0.5 ml of the PEG solution added [40 % (w/v) PEG 4000 in 0.4 M mannitol, 0.1 M Ca(NO<sub>3</sub>)<sub>2</sub>, (pH 7.0)]. The solutions are mixed gently and incubated for 30 minutes at room temperature (about 24°C) with occasional shaking.



1341473

1.4 ml of the wash solution is then added, and the contents of the tube gently mixed. The wash solution consists of 87 mM mannitol, 115 mM  $\text{CaCl}_2$ , 27 mM  $\text{MgCl}_2$ , 39 mM KCl, 7 mM Tris/HCl and 1.7 g/liter m-inositol, (pH 9.0). Four further 1.4 ml aliquots of wash solution are added at 4 minute intervals, with mixing after each addition. The tube is then centrifugated at about 60 g for about 10 minutes, and the supernatant discarded. The sedimented protoplasts are taken up in 1 ml KM-8p culture medium, and placed in a 10 cm petri dish. 10 ml of KM-8p medium containing 1.2 % (w/v) SeaPlaque® agarose is added. The protoplasts are evenly distributed throughout the medium, and the agarose allowed to gel.

- (b) Transformation is carried out as described in Example 7(a) except that the pH of the wash solution is adjusted to 5.6.
- (c) Transformation is carried out as described in Example 7(a) except that the pH of the wash solution is adjusted to 7.0.
- (d) Transformation is carried out as described in Example 7(a) to 7(c) except that the PEG used is PEG of MW 6'000.
- (e) Transformation is carried out as described in Example 7(a) to 7(c) except that the PEG used is PEG of MW 2'000.
- (f) Transformation is carried out as described in Example 7(a) to 7(c) except that the PEG used is PEG of MW 8'000.
- (g) Transformation is carried out as described in Example 7(a) to 7(f) except that in addition heat shock as described in Shillito et al, (1985) is used.
- (h) Transformation is carried out as described in Example 7(a) to 7(g) above except that the wash medium consists of 154 mM NaCl, 125 mM  $\text{CaCl}_2$ , 5 mM KCl, 5 mM glucose, pH to 6.0 with KOH.

- (i) Transformation is carried out as described in Example 7(a) to 7(g) above except that the wash medium consists of 0.2 M  $\text{CaCl}_2$ , 0.1 % (w/v) MES, pH to 6.0 with KOH.
- (j) Transformation is carried out as described in Example 7(a) to 7(g) above except that the wash medium consists of 0.2 M  $\text{CaCl}_2$ , 7 mM Tris/HCl, pH to 9.0 with KOH.

Example 8: Transformation of *Dactylis glomerata* L. protoplasts by electroporation or PEG treatment

- (a) Transformation is carried out as described in Examples 6 and 7 except that the pCIB709 plasmid DNA is restricted with restriction enzyme BglI before being used for transformation.
- (b) Transformation is carried out as described in Examples 6 and 7 except that the pCIB709 plasmid DNA is restricted with restriction enzyme HindIII before being used for transformation.

Example 9: Transformation of *Dactylis glomerata* L. protoplasts by electroporationh or PEG treatment

Transformation is carried out as described in Examples 6, 7 or 8, except that the protoplasts are treated at 45°C for about 5 minutes prior to distribution of the aliquots into tubes for transformation or after distribution of the aliquots, and before addition of the PEG.

Example 10: Selection of transformed colonies

- (a) The culture plates (petri dishes) containing the protoplasts are incubated for 10 days in the dark at about 25°C and then cut into 5 equal slices for "bead cultures" (Shillito et al, 1983). 4 of the slices are placed each into 20 ml SH-45 culture medium with 4 g/liter casein hydrolysate and 20 µg/ml hygromycin B. The fifth slice is put into 20 ml of the same medium but without hygromycin B as a non-selected control. After 4 to 5 weeks the putative transformed protoplast-derived cell colonies growing in hygromycin B are cut out of the agarose and placed into a 19 mm petri dish with 2 ml of liquid

SH-45 medium containing 20 µg/ml hygromycin B, which is agitated at about 50 rpm on an orbital shaker. After another 4 to 5 weeks all colonies which grow to make new suspensions are transferred into 125 ml erlenmeyer flasks and grown in a manner similar to the parent suspension culture, except that 20 µg/ml hygromycin B is included in the medium.

The new suspensions are subcultured every 1 to 3 weeks using SH-45 medium containing 4 g/liter casein hydrolysate and 20 µg/ml hygromycin B. Cells from these suspensions are also plated on solidified SH-30 medium containing 20 µg/ml hygromycin B and incubated at about 25°C in the dark. Calli grown from the plated cells are subcultured every two weeks onto fresh medium. The cells which grow in the presence of hygromycin B are presumed to be transformants.

- (b) Selection is carried out as described in Example 10(a) except that the protoplast-derived cell colonies growing in hygromycin B - containing medium are placed on agar plates of SH-30 medium containing 20 µg/ml hygromycin B and incubated at about 25°C in the dark.

Example 11: Regeneration of transformed *Dactylis glomerata* L. plants

Plants are regenerated from transformed callus as described in Example 4 for non-transformed material.

Example 12: Extraction of DNA from callus and leaf tissue

DNA is extracted from callus and leaves of regenerated plants using a modification of the CETAB method (Roger and Bendich, 1985). This method is described here for *Dactylis glomerata* L. but can be used as effectively on tissues of any other *Pooideae* plant. Other commonly used methods for DNA extraction can also be used to obtain DNA from this material.

Callus grown on SH-0 medium and SH-30 medium is frozen in dry ice, and then ground to a fine powder at liquid nitrogen temperature in a pestle and mortar. The resulting powder is transferred to a 5 ml polypropylene

1341473

centrifuge tube pre-cooled to liquid nitrogen temperature (2 g powder per tube). Care is taken that the powder never thaws out during the procedure. The powder is freeze-dried overnight, and then distributed into 2.2 ml Eppendorf tubes, < 0.5 ml powder per tube. 1 ml of CETAB extraction buffer is added to each tube, and they are incubated at 60°C for about 30 to 45 minutes. The tubes are allowed to cool to room temperature, and 1 ml of chloroform/isoamyl alcohol (24:1) added. After mixing, the solution is centrifuged for about 30 sec at 3'000 rpm in an Eppendorf centrifuge, and the water phase removed to a fresh tube. 1/10 volume of 10 % (w/v) CETAB solution is added, and the chloroform extraction is repeated. The water phase is removed to a fresh tube, and an equal volume of precipitation buffer added. The DNA and RNA precipitate at room temperature. After a period of about 30 minutes to 1 hour to allow precipitation, the tubes are again centrifuged, and the supernatant discarded. The precipitates are resuspended in high salt TE buffer (see below) at 65°C for about 30 minutes.

CETAB extraction buffer:

1 % (w/v) CETAB  
Tris pH 8.0 (50 mM)  
EDTA (10 mM)  
NaCl (0.7 M)  
0.5 % (w/v) PVP Mol.Wt.360,000  
[PVP: polyvinylpyrrolidine]

10 % CETAB:

10 % (w/v) CETAB  
NaCl (0.7 M)

Precipitation buffer:

1 % (w/v) CETAB  
Tris pH 8.0 (50 mM)  
EDTA (10 mM)

High salt TE:

Tris pH 8.0 (10 mM)  
EDTA (1 mM)  
NaCl (1 M)

TE buffer:

Tris pH 8.0 (10 mM)

EDTA (1 mM)

1/10 TE:

Tris pH 8.0 (1 mM)

EDTA (0.1 mM)

Example 13: Purification of the DNA

The DNA prepared as in Example 12 or any other suitable method can be purified by any of a number of known methods. Examples of suitable methods include but are not limited to: ethidium bromide CsCl gradient centrifugation, treatment with phenol/chloroform, and purification on a step gradient without ethidium bromide. Such methods are described in Maniatis *et al*, (1982).

(a) Purification by phenol/chloroform treatment

The nucleic acids from Example 12 above are precipitated with 2 volumes of cold ethanol (-20°C). The tubes are centrifuged for 2 to 3 minutes at 5'000 g. The supernatant is removed and the precipitate washed with 70 % ethanol and 100 % ethanol. The nucleic acids are partially dried in the airstream from a sterile flow bench. The DNA is dissolved overnight in 200 µl of 1/10 TE buffer. The DNA solution is transferred to an Eppendorf centrifuge tube and 10 µl of a 2 mg/ml solution of RNAase (boiled previously to deactivate DNAase), is added and the tubes incubated at 37°C for about 1 hour. 0.25 volume 5 M NaCl is added and the DNA precipitated by adding 0.4 volume of 30 % PEG (Mol.Wt. 6'000 to 8'000) containing 1.5 M NaCl and standing the tube at -20°C for about 1 hour. The tubes are centrifuged for 5 minutes, the supernatant removed, and the precipitate washed with cold absolute ethanol. After briefly drying in the air stream from a sterile flow bench, the pellet is resuspended in 0.3 ml TE buffer. The solution is extracted with phenol/chloroform/isoamyl alcohol (25:24:1) equilibrated with TE buffer, centrifuged for 30 s in an Eppendorf centrifuge, and the water phase transferred to a fresh tube. The solution is extracted with chloroform/isoamyl alcohol (24:1), centrifuged for 30 s, and the water phase removed to fresh tube. The chloroform extraction is repeated. A 1/10 volume of 3 M sodium acetate is added, followed by 2 volumes of ice cold absolute ethanol to precipitate the DNA. The precipitate is collected by centrifugation, and washed with

70 % and 100 % ethanol, dried briefly in a stream of sterile air, and dissolved in sufficient TE buffer to obtain a solution at 0.25 µg/ml to 1 µg/ml for use in Southern analysis.

(b) Purification on a step gradient without ethidium bromide

The nucleic acids are purified on a CsCl step gradient consisting of a bottom layer of 5.7 M CsCl in TE buffer, and a top layer of 1.0 M CsCl in TE. The nucleic acids are incorporated in the top layer. The tubes containing the gradient are centrifuged overnight in a swing-out rotor (e.g. Beckmann SW 50.1 at 45'000 rpm). The DNA is collected from the region of the interface, and RNA can be recovered from the bottom of the tube. The DNA is diluted with 2 volumes of water, and precipitated with 2 volumes of ice cold ethanol as in example (a) above. The precipitates are resuspended in TE buffer, precipitated again with ethanol, and used for Southern analysis.

Example 14: Detection of foreign DNA sequences in the genome of transformed *Dactylis glomerata* L. by Southern analysis

Southern hybridization analysis is performed essentially according to Maniatis et al, (1982). *Dactylis glomerata* L. DNA [purified according to Example 13(a) and 13(b)] is cut with restriction enzyme BamHI and 5 µg thereof are loaded per lane, and run on a 1 % agarose gel to separate the DNA on the basis of the fragment sizes. The gel is placed in 0.25 M HCl for about 20 minutes and then rinsed with H<sub>2</sub>O, and then placed in 0.4 M NaOH for 30 minutes. The DNA is transferred overnight to GeneScreen Plus<sup>R</sup> (NEN Res. Products, Cat. No. NEF 976, Lot. No. 330GP62) in the normal way (as described in the instruction booklet supplied with the product) using 0.4 M NaOH as the transfer buffer. After transfer overnight, the filter is removed, washed with 2 x SSC (0.3 M NaCl, 0.03 M Na citrate) for about 5 minutes, and then air dried. The blot is pre-hybridized for 4 hours at 65°C with buffer containing 10 g/liter Bovine Serum Albumin (Fat Free Sigma, Cat. No. A-4503), 7 % SDS, 1 mM NaEDTA, and 0.52 M Sodium Phosphate Buffer pH 7.0. A radioactively labelled probe is prepared by the random primer method using the IBI "Prime Time" labelling kit or any other suitable method, and separating the probe from the nucleotides in a spin column. The probe DNA consists of the fragment of pCIP709 containing the 35S promoter region and the aminoglycoside

1341473

phosphotransferase type IV structural gene region. Hybridization is allowed to proceed overnight at 65°C. The blot is then washed in four washes using SW wash buffer, the last two washes being carried out at 65°C. The blot is then washed for 2 hours in 0.2 x SSC containing 1 % SDS and 5 mM NaEDTA at 65°C. The wet blot is wrapped in food film (Saran-warp®) or any other suitable film, and exposed to a X-ray film (Kodak\* X-Omat AR film, Eastman Kodak, Rochester, NY 14650, Cat. No. 165 1454). On development, there is clear specific hybridisation of the probe to the DNA coming from callus and plants transformed with pCIP709. The pCIP709 DNA is clearly integrated into the high molecular weight DNA of the transformants.

SW hybridization buffer:

1 % (w/v) Bovine Serum Albumin (fat free)  
0.52 M Sodium phosphate pH 7.0  
7 % (w/v) SDS  
1 mM NaEDTA

Wash solution:

0.04 M Sodium phosphate pH 7.0  
1 mM NaEDTA  
1 % (w/v) SDS  
0.125 M NaCl

Example 15: Cryopreservation of callus cultures of *Dactylis glomerata* L.

- (1) Actively-growing callus of *Dactylis glomerata* L. is placed into liquid SH-0 medium. 0.5 g to 1 g of callus is typically placed into 20 ml of medium. The flask containing the callus is gently shaken and swirled, to disrupt and disperse the clumps of callus. The culture is then cooled on ice. The cryoprotectant solution is also cooled on ice.
- (2) An equal volume of cryoprotectant solution P is added over a period of 5 minutes, and the mixture is kept on ice for one hour. During this time, 1.0 ml aliquots are distributed to labeled precooled 1.8 ml plastic cryopreservation vials (Vanguard Cryos\* cryogenic vials, Sumitomo Bakelite Co. Ltd. Japan, Cat. No. MS4502), and kept on ice. Cryoprotectant Solution P consists of 1M Glycerol, 1 M L-Proline, 2M

\*Trade-mark

Dimethyl Sulfoxide (DMSO, Sigma, Cat. No. D2650, Lot No. 57F-8816) in water, pH 5.6, and is freshly prepared before each use (the glycerol/proline/water mixture may be stored frozen).

- (3) After the cells have been exposed to the cryoprotectant solution for a period of about 1 hour, the vials are immersed at the surface of a liquid bath which is at the temperature of 0°C. The bath may consist of ethanol or any other suitable coolant as known in the art. The bath is equipped with a stirring device to keep the coolant mixed, and is connected to an apparatus which can refrigerate the coolant at a controlled rate.
- (4) Once the vials are in the coolant, the temperature is reduced at the rate of approximately 0.5°C/minute. When the temperature reaches -40°C, the vials are plunged into liquid nitrogen and then stored in liquid nitrogen, either in the liquid itself or in the vapor above it, at a temperature not to exceed -100°C.

Example 16: Cryopreservation of embryogenic suspension culture cells of *Dactylis glomerata* L.

- (a) (1) A *Dactylis glomerata* L. suspension culture taken 2 to 10 days after subculture is cooled on ice. the cryoprotectant solution is normally also cooled on ice. The cryoprotectant consists of 1 M Glycerol, 1 M L-Proline, 2 M Dimethyl Sulfoxide (DMSO) in water, pH 5.6. The cryoprotectant solution is freshly prepared before each use or the glycerol/proline/water may be stored frozen.
- (2) The cryoprotectant is added to the suspension over a period of 5 minutes. The cells are left in the cryoprotectant on ice for one hour. During or after this time, aliquots are distributed to cryopreservation vials and kept on ice. The vials are then treated as described above for callus material in example 15.
- (b) Cryopreservation is carried out as described in Example 16(a) except that the cryoprotectant in step (1) consists of 1 M Glycerol, 1 M Sucrose and 2 M DMSO in water at pH 5.6.



1341473

Example 17: Recovery of Growing Cultures from Cryopreserved

*Dactylis glomerata* L.

- (a) (1) A vial prepared as in Example 15 is removed from the liquid nitrogen.
- (2) The vial is thawed by leaving it at room temperature until all the ice has melted.
- (3) The contents of the vial are spread onto SH-0 culture medium solidified with Gelrite® or agar. Typically, 0.5 ml of thawed culture is spread onto each 10 cm diameter petri plate containing 30 ml to 50 ml of medium. The solid medium is poured on a slant or a cavity is scooped out of the medium around its periphery in order to aid the drainage of remaining cryoprotectant away from the cells.
- (4) The material is incubated on the medium in the dark at 27°C. Growth is readily apparent in 1 to 4 weeks. Callus is then subcultured as for normal embryogenic callus as described above.
- (b) Recovery of growing cultures from cryopreserved *Dactylis glomerata* L. is carried out as described in Example 17(a), except that in step (2) the vial is thawed rapidly by agitating it in a water bath at about 40°C until all the ice has melted.

Example 18: Cryopreservation of *Zea mays* callus

Cryopreservation of actively growing *Zea mays* callus is carried out as described for *Dactylis glomerata* L. in Example 15.

Example 19: Cryopreservation of embryogenic suspension culture cells of

*Zea mays*

Cryopreservation of embryogenic suspension culture cells of *Zea mays* is carried out as described for *Dactylis glomerata* L. in Examples 16(a) and 16(b).

Example 20: Recovery of growing cultures from cryopreserved *Zea mays*

Recovery of growing cultures from cryopreserved *Zea mays* is carried out as described for *Dactylis glomerata* L. in Example 17(a) and 17(b).

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1341473

CLAIMS:

1. An embryogenic cell culture derived from graminaceous plants of the subfamily *Pooideae* from which protoplasts can be isolated, wherein the protoplasts  
5 regenerate cell walls, divide and form callus capable of being regenerated into plants.
2. The embryogenic cell culture according to claim 1 wherein said regenerated plants are fertile plants.
3. The embryogenic cell culture according to claim 1  
10 wherein the said *Pooideae* plants are grasses or small grain cereals.
4. The embryogenic cell culture according to claim 3 wherein the grasses are selected from the genera consisting of *Poa*, *Festuca*, *Lolium*, *Bromus*, *Trisetum*, *Agrostis*, *Phleum*,  
15 *Alpecurus* and *Dactylis*.
5. The embryogenic cell culture according to claim 3 wherein the small grain cereals are selected from the genera consisting of *Avena*, *Triticum*, *Secale* and *Hordeum*.
6. A protoplast or plant cell of graminaceous plants  
20 of the subfamily *Pooideae* capable of being regenerated into plants.
7. The protoplast or plant cell according to claim 6 wherein said regenerated plants are fertile plants.
8. The protoplast or plant cell according to claim 6  
25 having stably incorporated into their genome exogenous DNA.
9. The protoplast or plant cell according to claim 6 having stably incorporated into their genome exogenous DNA expressible in plants.

**E**

10. The protoplast or plant cell according to claim 6 derived from cell cultures.
11. The protoplast or plant cell according to claim 6 derived from embryogenic cell suspensions.
- 5 12. The protoplast or plant cell according to claim 6 derived from grasses or small grain cereals.
13. The protoplast or plant cell according to claim 12 wherein the small grain cereals are selected from the genera consisting of *Avena*, *Triticum*, *Secale* and *Hordeum*.
- 10 14. Callus derived from graminaceous plants of the subfamily *Pooideae*, which callus is regenerated from protoplasts or plant cells according to any one of claims 6 to 13 and is capable of being regenerated into plants.
15. The callus according to claim 14 wherein said  
15 regenerated plants are fertile plants.
16. The callus according to claim 14 wherein the *Pooideae* plants are grasses or small grain cereals.
17. The callus according to claim 16 wherein the small grain cereals are selected from the genera consisting of  
20 *Avena*, *Triticum*, *Secale* and *Hordeum*.
18. A cell culture derived from graminaceous plants of the subfamily *Pooideae*, which cell culture is regenerated from protoplasts or plant cells according to any one of claims 6 to 13 and is capable of being regenerated into  
25 plants.
19. The cell culture according to claim 18 wherein said regenerated plants are fertile plants.

20. The cell culture according to claim 18 wherein the *Pooideae* plants are grasses or small grain cereals.
21. The cell culture according to claim 20 wherein the small grain cereals are selected from the genera consisting  
5 of *Avena*, *Triticum*, *Secale* and *Hordeum*.
22. Cells of graminaceous plants of the subfamily *Pooideae* and cells of propagules thereof regenerated from an embryogenic cell culture according to any one of claims 1 to 5.
- 10 23. Cells of graminaceous plants of the subfamily *Pooideae* and cells of propagules thereof regenerated from protoplasts or plant cells according to any one of claims 6 to 13.
24. Cells of graminaceous plants of the subfamily  
15 *Pooideae* and cells of propagules thereof regenerated from a callus according to any one of claims 14 to 17.
25. Cells of graminaceous plants of the subfamily *Pooideae* and cells of propagules thereof regenerated from a cell culture according to any one of claims 18 to 21.
- 20 26. Cells of graminaceous plants of the subfamily *Pooideae* according to any one of claims 22 to 25, wherein said plants are fertile plants.
27. Cells of graminaceous plants of the subfamily *Pooideae* and cells of propagules thereof comprising  
25 protoplasts or plant cells containing stably incorporated into their genomes exogenous DNA.
28. Cells of graminaceous plants of the subfamily *Pooideae* and cells of propagules thereof according to

claim 27, wherein the stably incorporated exogenous DNA is expressible in plants or plant cells.

29. Cells of propagules according to any one of claims 22 to 28 wherein the propagules consist of plant material capable of being sexually or asexually, and *in-vivo* or *in-vitro* propagated.

30. Cells of propagules according to any one of claims 22 to 28 wherein the propagules consist of the protoplasts, cells, calli, tissues, organs, zygotes, embryos, pollen or seeds obtained from transgenic *Pooideae* plants.

31. Cells of the progeny of the plants of any one of claims 22 to 28.

32. A method of producing protoplasts of graminaceous plants of the subfamily *Pooideae*, which protoplasts are capable of being regenerated into plants, which process comprises:

(a) isolating tissue from suitable parts of *Pooideae* plants,

20 (b) culturing this tissue in a medium capable of inducing the formation of embryogenic callus and of embryos,

(c) periodically subculturing the embryogenic callus and embryos on fresh medium capable of sustaining continuous proliferation,

25 (d) isolating embryogenic cell clusters after 0 to 500 transfers, and

(e) removing the cell walls with suitable enzyme mixtures and isolating the resultant protoplasts.



33. The method according to claim 32 wherein the *Pooideae* protoplasts are capable of being regenerated into fertile plants.

34. The method according to claim 32 wherein the  
5 tissue of step (a) is isolated from the basal part of young inner leaves, immature sexual embryos, immature inflorescences, mature seeds or seedling tissue of *Pooideae* plants.

35. The method according to claim 34 wherein the  
10 tissue of step (a) is isolated from the youngest inner leaves.

36. The method according to claim 34 wherein the isolation of embryogenic cell clusters is carried out after 0 to 100 transfers.

15 37. The method according to claim 36 wherein the isolation of embryogenic cell clusters is carried out after 3 to 50 transfers.

38. A method of producing a cell culture of a graminaceous plant of the subfamily *Pooideae*, which cell  
20 culture is capable of being regenerated into plants, which method comprises:

(a) culturing in a suitable culture medium protoplasts capable of being regenerated into plants until they form cell colonies, and

25 (b) culturing said cell colonies or parts thereof on a medium suitable to promote cell culture formation, and

(c) isolating the resultant cell culture.

39. The method of producing a cell culture of a graminaceous plant of the subfamily *Pooideae*, which cell culture is capable of being regenerated into plants, which method comprises:

5 (a) culturing in a suitable culture medium protoplasts capable of being regenerated into plants for a time sufficient to promote culture formation until they form cell colonies, and

(b) isolating the resultant cell culture.

10 40. A method of producing a cell culture according to any one of claims 38 or 39, wherein the said cell culture is capable of being regenerated into fertile plants.

41. The method according to any one of claims 38 to 40 wherein the cell culture is a suspension culture.

15 42. The method according to any one of claims 38 to 40 wherein the cell culture is a callus culture.

43. The method according to claim 38 or 39 wherein step (a) is carried out in the presence of agarose as a solidifying agent.

20 44. The method according to claim 43 comprising liquefaction of the agarose solidified medium of step (a) or segmentation thereof, transferring the liquefied agarose medium or the segments of the agarose medium to a liquid nutrient medium, and culturing until cell colonies are  
25 formed.

45. The method according to claim 38 or 39 wherein the parts of the cell colonies in step (b) are cells released from the cell colonies.

1341473

46. The method according to claim 38 or 39 wherein the protoplasts contain stably integrated into their genomes exogenous DNA.

47. The method according to claim 46 wherein the  
5 integrated exogenous DNA is expressible in plant cells.

48. A method of regenerating graminaceous plants of the subfamily *Pooideae* from callus comprising:

(a) culturing callus of *Pooideae* plants, which callus is derived from protoplasts and is capable of being  
10 regenerated into plants on a medium capable of inducing embryo formation until embryos are formed,

(b) culturing the embryos on a medium suitable to induce them to mature and germinate, and

(c) culturing the resultant plantlets until  
15 sufficiently developed to be transferred to soil to form mature plants.

49. A method of regenerating fertile graminaceous plants of the subfamily *Pooideae* from callus, which process comprises:

20 (a) culturing callus of *Pooideae* plants, which callus is derived from protoplasts and is capable of being regenerated into fertile plants on a medium capable of inducing embryo formation until embryos are formed,

(b) culturing the embryos on a medium suitable to  
25 induce them to mature and germinate,

(c) culturing the resultant plantlets until sufficiently developed to be transferred to soil to form mature plants, and

(d) obtaining seed following controlled or open pollination.

50. The method according to claim 48 or 49 wherein the plant cells forming the callus contain stably integrated  
5 into their genome exogenous DNA.

51. The method according to claim 50 wherein the integrated exogenous DNA is expressible in plant cells.

52. A method for producing transgenic graminaceous plants of the subfamily *Pooideae*, which process comprises

10 (a) transforming protoplasts which are capable of being regenerated into whole plants with exogenous DNA,

(b) culturing the transformed protoplasts according to claim 38 or 39, and

(c) regenerating whole plants according to  
15 claim 48 or 49.

53. The method according to claim 52, wherein the said exogenous DNA comprises one or more chimeric genes which provide the transformed plant protoplasts, protoplast-derived tissues and finally protoplast-derived plants with  
20 valuable properties.

54. The method according to claim 52, wherein the said exogenous DNA comprises non-coding DNA sequences exhibiting regulatory functions.

55. The method according to claim 52, wherein a direct  
25 gene transfer method is used in step (a) for the transformation of protoplasts.

Application number / numéro de demande: 1341473

Figures: 1-2-3-4-6

Pages: 1-2-4

Unscannable items  
received with this application  
(Request original documents in File Prep. Section on the 10<sup>th</sup> floor)

Documents reçus avec cette demande ne pouvant être balayés  
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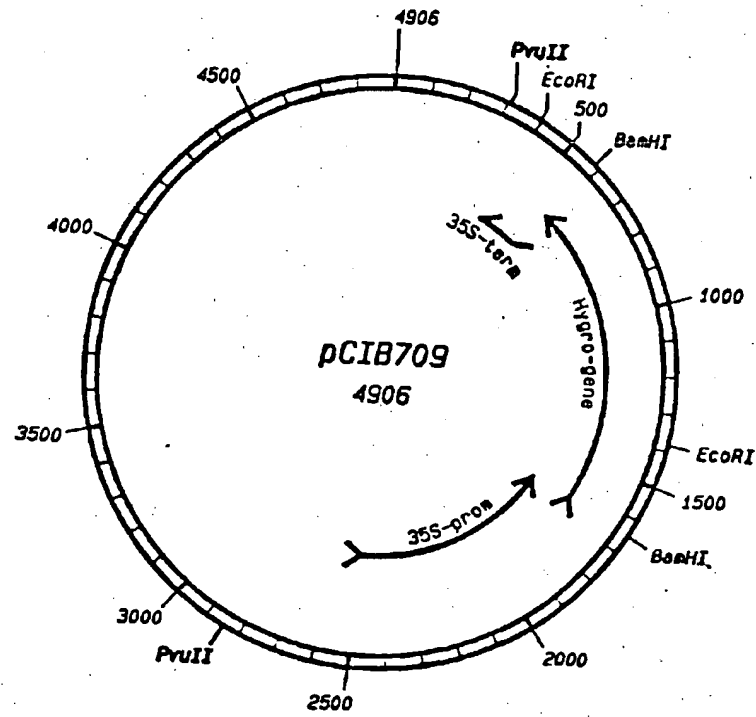


Figure 5.

Figure 7 NUCLEOTIDE SEQUENCE OF THE PLASMID PCIB709.

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1  TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG
51  GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG
101 TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG
151 CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA
201 CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCGCCATT
251 CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT
301 TACGCCAGCT GCGGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA
351 ACGCCAGGGT TTTCCAGTC ACGACGTTGT AAAACGACGG CCAGTGAATT
401 CGAGCTCGGT ACCCGGAGAT CTGGATTTTA GTACTGGATT TTGGTTTTAG
451 GAATTAGAAA TTTTATTGAT AGAAGTATTT TACAAATACA AATACATACT
501 AAGGGTTTCT TATATGCTCA ACACATGAGC GAAACCCTAT AAGAACCCTA
551 ATTTCCCTTA TCGGGAAACT ACTCACACAT TAGGATCCCG GTCGGCATCT
601 ACTCTATTCC TTTGCCCTCG GACGAGTGCT GGGGCGTCGG TTTCCACTAT
651 CGGCGAGTAC TTCTACACAG CCATCGGTCC AGACGGCCGC GCTTCTGCGG
701 GCGATTGTG TACGCCCGAC AGTCCCGGCT CCGGATCGGA CGATTGCGTC
751 GCATCGACCC TCGGCCCAAG CTGCATCATC GAAATTGCCG TCAACCAAGC
801 TCTGATAGAG TTGGTCAAGA CCAATGCCGA GCATATACGC CCGGAGCCGC
851 GCGGATCCTG CAAGCTCCGG ATGCCCTCCG TCGAAGTAGC GCGTCTGCTG
901 CTCCATACAA GCCAACCACG GCCTCCAGAA GAAGATGTTG GCGACCTCGT
951 ATTGGGAATC CCCGAACATC GCCTCGCTCC AGTCAATGAC CGCTGTTATG
1001 CGGCCATTGT CCGTCAGGAC ATTGTTGGAG CCGAAATCCG CGTGCACGAG

1051 GTGCCGGA CTGCGGGCAGT CCTCGGCCCA AAGCATCAGC TCATCGAGAG
1101 CCTGCGCGAC GGACGCACTG ACGGTGTCGT CCATCACAGT TTGCCAGTGA
1151 TACACATGGG GATCAGCAAT CGCGCATATG AAATCACGCC ATGTAGTGTA
1201 TTGACCGATT CTTGCGGTC CGAATGGGCC GAACCCGCTC GTCTGGCTAA
1251 GATCGGCCGC AGCGATCGCA TCCATGGCCT CCGCGACCGG CTGCAGAACA
1301 GCGGGCAGTT CGGTTTCAGG CAGGTCTTGC AACGTGACAC CCTGTGCACG
1351 GCGGGAGATG CAATAGGTCA GGCTCTCGCT GAATTCCCCA ATGTCAAGCA
1401 CTTCCGGAAT CGGGAGCGCG GCCGATGCAA AGTGCCGATA AACATAACGA
1451 TCTTTGTAGA AACCATCGGC GCAGCTATTT ACCCGCAGGA CATATCCACG
1501 CCCTCCTACA TCGAAGCTGA AAGCACGAGA TTCTTCGCCC TCCGAGAGCT
1551 GCATCAGGTC GGAGACGCTG TCGAACTTTT CGATCAGAAA CTTCTCGACA
1601 GACGTCGCGG TGAGTTCAGG CTTTTTCATA TCTCATTGCC CCCCGGGATC
1651 CTTATAGAGA GAGATAGATT TGTAAGAGAGA GACTGGTGAT TTCAGCGTGT
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1701 CCTCTCCAAA TGAAATGAAC TTCCTTATAT AGAGGAAGGG TCTTGCGAAG  
1751 GATAGTGGGA TTGTGCGTCA TCCCTTACGT CAGTGGAGAT ATCACATCAA  
1801 TCCACTTGCT TTGAAGACGT GGTGGGAACG TCTTCTTTT CCACGATGCT  
1851 CCTCGTGGGT GGGGGTCCAT CTTTGGGACC ACTGTCGGCA GAGGCATCTT  
1901 GAACGATAGC CTTTCCTTTA TCGCAATGAT GGCATTGTGA GGTGCCACCT  
1951 TCCTTTTCTA CTGTCTTCAT GATGAAGTGA CAGATAGCTG GGCAATGGAA  
2001 TCCGAGGAGG TTTCCGAAA TTACCCTTG TTGAAAAGTC TCAATTGCCC  
  
2051 TTTGGTCTTC TGAGACTGTA TCCTTGATAT TTTTGGAGTA GACCAGACTG  
2101 TCGTGCTCCA CCATGTTGAC GAAGATTTTC TTCTTGTCAT TGAGTCGTAA  
2151 GAGACTCTGT ATGAACGTGT CGCCAGTTT CACGGCGAGT TCTGTTAGAT  
2201 CCTCGATTG AATCTTTGAC TCCATGGCCT TTGATTCACT AGGAACACT  
2251 TTTTATAGAGA CTCCAATCTC TATTACTTGC CTGGTTTAT GAAGCAAGCC  
2301 TTGAATCGTC CATACTGGAA TAGTACTTCT GATCTTGGAG AAATATATCT  
2351 TTCTCTGTGT TCTTGATGCA GTTAGTCCTG AATCTTTTGA CTGCATCTTT  
2401 AACCTTCTTG GGAAGGTATT TGATCTCCTG GAGATTATTA CTCGGGTAGA  
2451 TCGTCTTAAT GAGACCTGCT GCGTAGGCCT CTCTAACCAT CTGTGGGTTA  
2501 GCGTCTTTC TGAATTTGAA GAGGCTAATC TTCTCATTAT CAGTGGTGAA  
2551 CATAGTATCG TCACCTTCAC CGTCGAACTT TCTTCCTAGA TCGTAGAGAT  
2601 AGAGGAAGTC GTCCATTGTA ATCTCCGGG CAAAGGAGAT CCTCTAGAGT  
2651 CGACCTGCAG GCATGCAAGC TTGGCGTAAT CATGGTCATA GCTGTTTCCT  
2701 GTGTGAAAT GTTATCCGCT CACAATTCCA CACAACATAC GAGCCGGAAG  
2751 CATAAAGTGT AAAGCCTGGG GTGCCTAATG AGTGAGCTAA CTCACATTA  
2801 TTGCGTTGCG CTCACTGCCC GCTTCCAGT CGGGAAACCT GTCGTGCCAG  
2851 CTGCATTAAT GAATCGGCCA ACGCGCGGG AGAGGCGGTT TCGGTATTGG  
2901 GCGCTCTTCC CGTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTGGG  
2951 TCGGCGGAGC GGTATCAGCT CACTCAAAG CGGTAATACG GTTATCCACA  
  
3001 GAATCAGGGG ATAACGCAGG AAAGAAGATG TGATCAAAAG GCCAGCAAAA  
3051 GGCCAGGAAC CGTAAAAAG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC  
3101 GCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA  
3151 AACCCGACAG GACTATAAAG ATACCAGCG TTTCCCCTG GAAGCTCCCT  
3201 CGTGCGCTCT CTTGTTCCGA CCTGCGCGT TACCGGATAC CTGTCCGCT  
3251 TTCTCCCTTC GGAAGCGTG GCGCTTCTC AATGCTCACG CTGTAGGTAT  
3301 CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC  
3351 CCCCCTCAG CCCGACCGCT GCGCCTTATC CGGTAACAT CTTCTTGAGT  
3401 CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC  
3451 AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GTCACAGAGT TCTTGAAGTG



3501 GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC  
3551 TGCTGAAGCC AGTTACCTTC GAAAAAAGAG TTGGTAGCTC TTGATCCGGC  
3601 AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT  
3651 TACGCGCAGA AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG  
3701 GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG  
3751 AGATTATCAA AAAGGATCTT CACCTAGATC CTTTAAAT AAATGAAG  
3801 TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC  
3851 AATGCTTAAT CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTGCTTCA  
3901 TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACACGA TACGGGAGGG  
3951 CTTACCATCT GGGCCCAAGT CTGCAATGAT ACCGCGAGAC CCACGCTCAC  
  
4001 CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGGCAGCGC  
4051 AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG  
4101 CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG  
4151 TGGCCATTGC TACAGGCATC GTGGTGTAC GCTCGTCGTT TGGTATGGCT  
4201 TCATTACAGT CCGGTTCCCA AGCATCAAGG CGAGTTACAT GATCCCCCAT  
4251 GTTGTGCAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTGAGAA  
4301 GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT  
4351 TCTCTTACTG TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA  
4401 CTCAACCAAG TCATTCTGAG AATAGTGATC GTGGCGACCG AGTTGCTCTT  
4451 GCGCGGCGTC AATACGGGAT AATACGCGC CACATAGCAG AACTTTAAAA  
4501 GTGCTCATCA TTGGAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT  
4551 ACCGCTGTTG AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT  
4601 CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAACAGGA  
4651 AGGCAAAATG CCGCAAAAAA GGAATAAGG GCGACACGGA AATGTTGAAT  
4701 ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT  
4751 GTCTCATGAG CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA  
4801 GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG TCTAAGAAAC  
4851 CATIATTATC ATGACATTAA CCTATAAAAA TAGGCGTATC AGCAGGCCCT  
4901 TTCGTC